

RESEARCH ARTICLE

Cannabinoid Receptors Are Overexpressed in CLL but of Limited Potential for Therapeutic Exploitation

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Abbreviations: CLL, chronic lymphocytic leukemia; CNB, (-)-cannabidiol; CNR1/2, CB1/2, cannabinoid receptor 1/2; HL, Hodgkin lymphoma; MCL, mantle

Abstract

The cannabinoid receptors 1 and 2 (CNR1&2) are overexpressed in a variety of malignant diseases and cannabinoids can have noteworthy impact on tumor cell viability and tumor growth. Patients diagnosed with chronic lymphocytic leukemia (CLL) present with very heterogeneous disease characteristics translating into highly differential risk properties. To meet the urgent need for refinement in risk stratification at diagnosis and the search for novel therapies we studied CNR expression and response to cannabinoid treatment in CLL. Expression levels of CNR1&2 were determined in 107 CLL patients by real-time PCR and analyzed with regard to prognostic markers and survival. Cell viability of primary CLL cells was determined in suspension and co-culture after incubation in increasing cannabinoid concentrations under normal and reduced serum conditions and in combination with fludarabine. Impact of cannabinoids on migration of CLL cells towards CXCL12 was determined in transwell plates. We found CNR1&2 to be overexpressed in CLL compared to healthy B-cells. Discriminating between high and low expressing subgroups, only high CNR1 expression was associated with two established high risk markers and conferred significantly shorter overall and treatment free survival. Viability of CLL primary cells was reduced in a dose dependent fashion upon incubation with cannabinoids, however, healthy cells were similarly affected. Under serum reduced conditions, no significant differences were observed within suspension and co-culture, respectively, however, the feeder layer contributed significantly to the survival of CLL cells compared to suspension culture conditions. No significant differences were observed when treating CLL cells with cannabinoids in combination with fludarabine. Interestingly, biologic activity of cannabinoids was independent of both CNR1&2 expression. Finally, we did not observe an inhibition of CXCL12-induced migration by cannabinoids. In contrast to other tumor entities, our data suggest a limited usability of cannabinoids for CLL therapy. Nonetheless, we could define CNR1 mRNA expression as novel prognostic marker.

cell lymphoma; PBMC, peripheral blood mononuclear cells; RM, R-(+)-methanandamide.

Introduction

Cannabinoids, the active components of the hemp plant *Cannabis sativa*, have been used for centuries for medical and recreational purposes. These compounds exert their activity by binding the cannabinoid receptors.

Cannabinoid receptors 1 and 2 (CNR1/CNR2; CB1/2) belong to the group of G protein-coupled receptors (GPR) and are part of the endocannabinoid system. The native ligands of the two receptors, such as 2-arachidonoyl glycerol, are produced on demand and fulfill a variety of functions. Although other receptors have been described for the endocannabinoid system [1], CB1&2 still are the two receptors for which most knowledge has been gathered.

CNR1 is primarily expressed in the brain, CNR2 in cells and tissues of the immune system, but both receptors have also been found outside these main sites of expression [2–4]. The two receptors appear to mediate similar responses and to exert overlapping influences, they seem to interact with respect to inflammatory and neurologic/psychotic conditions [5–9], and are involved in migration of cells of different origin under different physiological states [10–12]. Thus, 2-arachidonoyl glycerol acts as chemo-attractant for both immature and mature B-cells via CB2 [13–16] and appears to interfere with CXCR4 expression and/or CXCL12 induced migration [14]. Similar migratory effects were reported also for other cannabinoids [17], although the extent of these effects seems to be variable [18, 19].

Overexpression of both receptors has been found both in solid tumors and hematologic malignancies and cannabinoids were shown to inhibit cell migration, angiogenesis, to reduce proliferation and viability, to induce apoptosis *in vitro* and reduce tumor burden *in vivo* [4, 20–32]. The sensitivity of mantle cell lymphoma (MCL), chronic lymphocytic leukemia (CLL), and Hodgkin lymphoma (HL) cell lines to cannabinoids was linked to the overexpression of CNR1 and/or CNR2 [23, 33, 34]. While some of these reports used relatively selective agonists like ACEA (CB1), JWH133, or JWH015 (both CB2) [25, 30, 35–37], in the majority of studies cannabinoids were tested which appear to display broader activity on G-coupled protein receptors [23, 28, 31, 33, 38]. Thus cannabidiol acts as CB1 antagonist, CB2 inverse agonist, GPR55 antagonist, and agonist for the VR1 vanilloid and the μ -opioid receptors [39, 40], (R)-(+)-methanandamide as CB1 agonist but also displays activity at vanilloid receptors and other G-protein coupled receptors and ion channels [1, 41].

In solid tumors, expression of the two cannabinoid receptors has been linked to patient outcome. In hepatocellular carcinoma and mobile tongue squamous cell carcinoma, both CB1 and CB2 overexpression was associated with good prognosis [42, 43]. In contrast, CB1 expression was reported to be a marker of bad prognosis in prostate and colorectal cancer [44–46], while CB2 was shown to be a poor prognostic marker in colon cancer [32] and was linked to poorer survival in HER2 positive breast cancer and squamous cell carcinoma of head and neck [47, 48]. Whether increased expression of one of the two receptors or both has clinical implications in hematologic malignancies appears to be variable [49–51].

The development of targeting drugs in recent years has greatly improved therapeutic options in CLL [52–54]. However, it is not known how long targeting molecules will display their potential before patients develop resistances and/or progress. In this line, several reports already discussed genetic changes developing during treatment with such compounds [55, 56]. CLL, like other malignancies, consists of a pool of malignant clones [57–59], which develop and evolve during disease course. Changes in this clonal landscape may occur during treatment and/or due to the acquisition of resistance mutations. Therefore, there still is an urgent need for agents which can be used for combination therapy as well as supportive regimens to increase treatment options and to improve patient care.

Based on the reported aberrant expression of cannabinoid receptors in neoplasms, we studied the expression of the two receptors in CLL patients analyzing it in relation to clinical parameters to determine their usability for prognosis. Additionally, considering the versatile aspects of cannabinoid actions, we evaluated the potential of cannabinoids for use in CLL therapy.

Materials and Methods

Patient material

Peripheral blood samples were collected from 107 consecutive patients diagnosed with CLL at the Division of Hematology and Hemostaseology of the General Hospital in Vienna, Austria. All patients and the four healthy volunteers included in the study signed informed consent according to the Declaration of Helsinki. The study was approved by the Ethics Committee of the Medical University of Vienna (approval Nr: 1011/2012). Clinical characteristics for the patients used in mRNA expression analysis are listed in [S1 Table](#).

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll (Biocoll, Biochrom, Berlin, Germany) separation following standard procedures, cells were stored viable in liquid nitrogen. For protein analyses, 1×10^7 PBMCs were centrifuged, the supernatant was discarded and cell pellets were stored at -80°C . For RNA extraction, primary cells were stored at -80°C in TRIzol (Life Technologies Ltd, Paisley, UK). B lymphocytes from healthy donors were isolated using the EasySep™ Human B Cell Enrichment Kit without CD43 Depletion (STEMCELL Technologies SARL, Grenoble, France) following the manufacturer's protocol.

Reagents

Cannabinoids used in cytotoxicity and migration experiments were ACEA (CB1 agonist), AM251 (CB1 antagonist, μ -opioid receptor antagonist, GPR55 agonist, ion channel activation), AM630 (CB2 antagonist/inverse agonist, weak partial CB1 agonist, ion channel activation), (-)-cannabidiol (CNB) (GPR55 & weak CB1 antagonist, CB2 inverse agonist, weak agonist at VR1 vanilloid receptors, and modulator at opioid receptors), JWH133 (CB2 antagonist), and R-(+)-methanandamide (RM) (CB1 agonist, activity against GPR and ion channels) purchased from TOCRIS Bioscience (Bristol, UK). Fludarabine (2-Fluoroadenine-9- β -D-arabinofuranoside, SIGMA-ALDRICH, Vienna, Austria) served as control in cytotoxicity experiments, CXCL12/SDF-1 α (R&D SYSTEMS, Abingdon, UK) and CXCR4 antagonist AMD3100 octahydrochloride (TOCRIS Bioscience, Bristol, UK) were controls in migration experiments.

Primary antibodies

The following cannabinoid receptor directed antibodies were tested:

Anti-CB1 antibodies: Cat.No. PA1-745, Thermo Fisher Scientific Inc., Waltham, MA, USA; Cat.No. GTX100517, GeneTex Inc., Irvine, CA, USA; Cat.No. AF1185a, Abgent, San Diego, CA, USA.

Anti-CB2 antibodies: Cat.No. AF1575a, monoclonal, Abgent, San Diego, CA, USA; Cat.No. AF1186a, polyclonal, Abgent, San Diego, CA, USA; Cat.No. PA1-744, Thermo Fisher Scientific Inc., Waltham, MA, USA; Cat.No. GTX100391, GeneTex Inc., Irvine, CA, USA.

Cat.No. CB1001, anti-GAPDH monoclonal antibody, Calbiochem/EMD Millipore, Merck, Darmstadt, Germany.

Recombinant proteins for Western blots: CB1 and CB2 human recombinant proteins (Cat. Nos. H00001268-G01 and H00001269-G01; Abnova, Taipei City, Taiwan).

Secondary antibodies

IRDye 680 conjugated goat anti-mouse polyclonal IgG (H+L) (Cat.No. 926–32220), and IRDye 800CW conjugated goat anti-rabbit polyclonal IgG (H+L) (Cat.No. 926–32211), both purchased from LI-COR (Bad Homburg, Germany).

RNA extraction and cDNA synthesis

RNA was extracted from samples of CLL patients and healthy donors using TRIzol, RNA was dissolved in 10 μ l DEPC water, and the amount of isolated RNA measured. Two μ g of RNA were used for cDNA synthesis (all products from Promega Corporation, Madison, USA), cDNA was stored at -20°C until real time PCR.

Real time PCR

Real time PCR was carried out using TaqMan Gene Expression Assays on demand for Cannabinoid receptors 1 and 2 (Cat.Nos. Hs00275634_m1 and Hs00361490_m1, Life Technologies Ltd, Paisley, UK) according to the manufacturer's protocol. Applied Biosystems[®] Human ACTB (Cat.No. 4326315E) served as housekeeping gene. Samples were run in duplicates on an ABI Prism 7000 Sequence Detector and analyzed using the SDS Software. For calculation of mRNA expression, the $\Delta\Delta\text{Ct}$ -method was used [60], for which CD19 sorted pooled healthy B cells were set as 1.

Protein isolation and Western Blot

Proteins from patient samples were prepared using RIPA Buffer, concentration was determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, USA). Sixty μ g of protein per lane were separated by 10% SDS PAGE and transferred to PVDF Immobilon-FL Transfermembrane (pore size 0.45 μm ; Merck Millipore, Billerica, US). Membranes were incubated for 16 hours at 4°C with the primary antibody, were washed 3 times for 5 minutes with PBS (PAA laboratories, Pasching, Austria) + 0.1% Tween20 (Sigma Aldrich, St. Louis, MO, USA), and subsequently incubated 45 minutes at room temperature (RT) in the dark with the secondary antibody before detection on an Odyssey Imager (LI-COR, Bad Homburg, Germany)

Cell culture

All cells were cultured under standard conditions (95% humidity, 5% CO_2 , 37°C). M2-10B4 (purchased from the American Type Culture Collection; <http://www.lgcstandards-atcc.org>) were kept in RPMI1640 containing 10% fetal calve serum (FCS) and 1% Penicillin/Streptomycin (PS) as were primary CLL cells and healthy PBMC. All reagents were purchased from Life Technologies (Carlsbad, CA, USA).

Drug incubations and viability tests

All experiments were performed using medium without phenol red. Viability was determined using the CellTiter-Blue[®] Viability Assay (Promega, Madison, WI, USA) following the manual. Experiments with M2-10B4 were done in triplicates and repeated twice. Drug incubations with primary cells were done in triplicates using 10–18 samples from CLL patients and 2–3 healthy donors (see respective figures for the number of samples included in each incubation). Concentrations ranged between 0 and 100 μM , the optimal range having been determined in preliminary experiments. Concentrations are indicated for each compound and experiment in the respective figures. Cells incubated with vehicle only served as controls, vehicle did not

exceed 1% of experimental volume. Viability was calculated as the mean of all replicates and was normalized to vehicle control (set as 100%).

Primary cells from both CLL patients and healthy donors were tested in suspension culture for 48 h at 37°C at concentrations of 3×10^6 cells/ml in 96 well plates before viability was measured. For co-culture incubations of CLL cells, 2×10^5 M2-10B4 cells were seeded in 12 well plates and incubated for 24 h under standard conditions. On the next day, primary CLL cells were transferred to the wells at a concentration of 3×10^6 cells/ml. Compounds were added and plates were incubated for 48 h at 37°C. Then, CLL cells were transferred to 96 well plates before carrying out the CellTiter Blue assay.

To determine whether CB1&2 agonists and antagonists exert an additive cytotoxic effect when combined with fludarabine, CLL primary cells ($n = 5$) were prepared for co-culture incubation as described above. Cells were treated with increasing concentrations of ACEA and JWH133, and single concentrations of AM251, AM630, and CNB. Using the same setup, cells were pre-incubated with cannabinoids for 30 minutes before 5 μ M fludarabine was added. For comparison, primary cells were incubated in co-culture with vehicle alone, fludarabine (5 μ M) alone, AMD3100 (0,629 mM) alone, and were pre-treated with AMD3100 for 30 minutes before fludarabine was added. Plates were left for 48 h at 37°C before viability was determined.

To evaluate a potential effect of serum on cannabinoid toxicity, primary CLL cells from 5 patients were tested at different FCS concentrations (1, 2.5, 5, and 10%) both in suspension and in co-culture with M2-10B4. Preparation of experiments and determination of viable cells were done as described above.

Migration assays

CLL cells of 7 patients were sorted using the Human B cell Enrichment Kit (CatNo.8804-6818-74, Affymetrix eBiosciences, San Diego, CA, USA) following the manufacturer's recommendations. Resulting CD19+CD5+ cell purity was $97.7\% \pm 1.04$. Cells (5×10^6 cells/ml) were incubated with AM251, AM630, JWH133, ACEA, or with a combination of antagonist + agonist. Incubations with vehicle, with AMD3100, and without CXCL12 served as controls. Compound concentrations were chosen to exert minimal cytotoxic effects on cells. A list of concentrations and combinations can be found in [S2 Table](#).

Six hundred μ l RPMI1640 without phenol red were pipetted into the bottom chambers of 24-well polycarbonate membrane transwell plates (6.5 mm diameter, 5.0 μ m pore size; Corning Inc., Corning, NY, USA), CXCL12 (0,1 μ g/ml) or vehicle (0.1% PBS with 0.1% BSA) was added to the medium. After addition of transwell inserts, 100 μ l of treated cells were transferred to the upper chamber. Migration was allowed to occur for 4h at 37°C. The contents of both the upper and the bottom wells were transferred to separate tubes, centrifuged, pellets were resuspended in PBS, and cells were counted by FACS on a BD FACS Canto II Flow Cytometer.

A similar set of experiments was done with unsorted PBMC from CLL patients ($N = 5$) for which cells were pre-incubated with agonist, antagonist, or the combination of both before being transferred to the transwell plates. Incubation time was 0.5 h for antagonist, 1 h for agonist, and 1.5 h for antagonist plus agonist. An overview of these combinations can also be found in [S2 Table](#). In this setting, cells were counted in a Neubauer counting chamber.

Migration is shown as means + SD of the migration index which was calculated as the number of cells migrated in the presence of compound divided by the number of cells migrated in the presence of vehicle [[61–63](#)].

Statistical methods

CNR1 and 2 expressions are given as median, quartiles and range. The median was set as cut-off for CNR expression to distinguish CNR high and low expression groups. The impact of CNR mRNA expression on survival was tested by Kaplan-Meier plots (*P* values from log-ranks tests) and quantified by hazard-ratios in univariate and multivariable Cox regression analyses. Progression free survival was calculated from first treatment to progression, overall survival was measured from date of first diagnosis to follow up and treatment free survival was calculated from date of first diagnosis till first treatment or follow up. Prognostic markers were compared between groups using Chi²-tests. Pairwise comparison of migration experiments and viability assays was done by t-test. For this, IC₅₀ values were compared between CLL suspension and co-culture at the same compound concentration, between CLL suspension culture and healthy donors in suspension at the same compound concentration, and—for reduced serum conditions—between CLL suspension and co-culture at the same compound and serum concentrations. In addition, again for reduced serum conditions, IC₅₀ values were compared within CLL suspension and co-culture experiments, respectively, at the same compound but at different serum concentrations. Pearson correlation and linear regression analyses were performed to determine a potential association between IC₅₀ values and CNR mRNA expression. *P*-values ≤ 0,05 were considered statistical significant. Computations were performed using SPSS or GraphPad Prism.

Results

CNR mRNA and protein expression in CLL

CNR1 mRNA expression ranged from 0.00 to 140.39 with a median expression of 1.52. CNR2 mRNA had a median expression of 3.77, ranging from 0.06 to 26.54. Receptor expression of healthy, CD19 sorted B-cells was set as 1.

Using the median expression as cut-off, patients were split into high and low expressing groups for both receptors. Medians (ranges) were 0.23 (0.00–1.41) and 7.16 (1.52–140.39) for the CNR1 low and high expressing groups, and 2.35 (0.06–3.72) vs. 5.41 (3.77–26.54) for CNR2 low and high expressing groups, respectively. The number of patients in the 4 groups were 31 in the double low expressing group, 44 in the mixed group (one receptor high, the other low expressing), and 32 were double high expressers.

In order to determine whether receptor protein expression follows mRNA expression, we carried out Western blots using different commercially available antibodies (listed in [Methods](#)). In these experiments, we included recombinant proteins for both receptors as positive controls for CB1&2 expression. Despite testing different protein extraction and transfer protocols, we were not able to a) determine specific bands, and b) detect differences in receptor expression (data not shown). We concluded that the antibodies used in our experiments were unspecific, most likely showing crossreactivity with both receptors and/or detecting other proteins concomitantly. Subsequently, protein data were excluded from any analysis.

CNR1 but not CNR2 mRNA is a prognostic marker in CLL

Next, we analyzed patient data with regard to high and low mRNA expression of CNR1 and CNR2, respectively. We found that, based on the level of CNR1 expression, patient characteristics were significantly different for two prognostic markers. Thus, persons displaying high CNR1 mRNA expression were more likely to have unmutated IgHV genes (54.3 vs. 32.7%; *p* = 0.033), or high CD38 expression (42.6 vs. 21.6%; *p* = 0.026) ([Table 1](#)). In addition, CNR1 high expressing patients had a significantly shorter overall and treatment free survival (OS;

Table 1. Comparison of patient characteristics between CNR1 high and low mRNA expressing groups.

	CNR1 low	CNR1 high	p-value
Age at diagnosis [years]			
Median (Range)	59 (25–85)	63 (39–82)	
Sex [%]			
Female:Male	47.2:52.8	38.9:61.1	
CD19+CD5+ cells [%]			
Median (Range)	79.0 (28–97)	86.0 (42–97)	
Mutational status* [%]			
Unmutated	32.7	54.3	0.033
Mutated	67.3	45.7	
CD38 [%]			
Low < 30	78.4	57.4	0.026
High ≥ 30	21.6	42.6	
Median (Range)	5.0 (0–91)	23.0 (0–85)	
Binet at diagnosis [%]			
A	88.5	81.1	0.296
B/C	11.5	18.9	
Lymphocyte doubling time [%]			
Low < 1 year	18.0	30.4	0.154
High ≥ 1 year	82.0	69.6	
Del13q [%]			
Unmutated < 5.0	46.0	50.0	0.686
Mutated ≥ 5.0	54.0	50.0	
Del11q [%]			
Unmutated < 8.6	84.0	71.2	0.121
Mutated ≥ 8.6	16.0	28.8	
Del17p [%]			
Unmutated < 10.2	90.0	92.3	0.681
Mutated ≥ 10.2	10.0	7.7	
Tris12 [%]			
Unmutated < 3.7	86.0	92.3	0.305
Mutated ≥ 3.7	14.0	7.7	
Rearr14q [%]			
Unmutated < 3.0	91.8	81.6	0.136
Mutated ≥ 3.0	8.2	18.4	

*Cut-off 98% germline homology. Abbreviations: Del, deletion; Tris, trisomy; Rearr, rearrangement. P-values (Pearson Chi-Square) reaching statistical significance are bold.

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TFS) (OS: HR = 8.615; 95% CI 1.947–38.112; p = 0.001; TFS: HR = 2.770; 95% CI 1.603–4.785; p < 0.0001; Fig 1). No such difference could be observed for progression free survival (PFS; p = 0.171 (log rank)). Of note, CNR mRNA expression did not correlate with the fraction of CD19+CD5+ cells. In multivariate analyses, the influence of CNR1 mRNA expression on OS was independent from CD38 expression, IgHV mutational status, clinical stage B & C, and lymphocyte doubling time, but not so for TFS.

In contrast, no significant differences could be observed between CNR2 high and low expression groups. CNR2 mRNA expression levels were not associated with any of the established prognostic markers, nor did high expression translate into shorter OS, TFS, or PFS

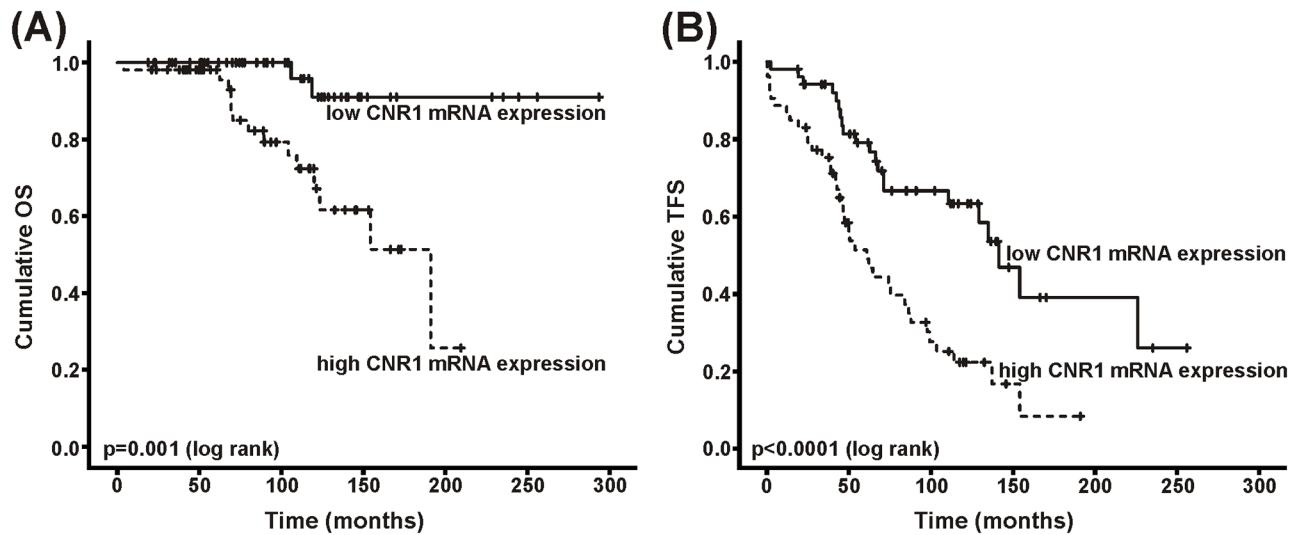


Fig 1. High CNR1 mRNA expression (≥ 1.52) confers significantly shorter survival in CLL patients ($n = 107$). (A) High expressing patients had a mean overall survival (OS) of 153 months compared to 277 months in low expressing patients ($p = 0.001$). (B) The mean treatment free survival (TFS) was 75 months in the CNR1 high group vs. 150 months in the CNR1 low group ($p < 0.0001$).

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($p = 0.763$, $p = 0.229$, $p = 0.089$, respectively (log rank)). Patient characteristics in relation to CNR2 high and low expression are listed in [S3 Table](#), Kaplan Meier Analyses for OS and TFS are shown in [S1 Fig](#).

Impact of cannabinoids on viability of primary cells

A dose dependent reduction of viability could be observed in CLL primary cells with increasing concentration of drug both in suspension and co-culture experiments ([Fig 2](#)).

This reduction was more pronounced in suspension culture compared to co-culture for the selective CB1 antagonist AM251 ($p = 0.0431$) compared to the selective CB1 agonist ACEA ($p = 0.1855$) and more pronounced for the selective CB2 agonist JWH133 ($p = 0.0527$) compared to the selective CB2 antagonist/inverse agonist AM630 ($p = 0.1353$). No differences between the two culture conditions could be observed for (R)-(+)-methanandamide (RM) (CB1 agonist, activity against vanilloid and other G-protein coupled receptors and ion channels) and (-)-cannabidiol (CNB) (weak CB1 antagonist, CB2 inverse agonist, interacting also with GPR55, vanilloid receptors and opioid receptors) ($p = 0.1464$ and $p = 0.6549$, respectively). These data suggest a protective effect of the fibroblasts in incubations with AM251 and JWH133. The loss of this effect, at least for RM, CNB, and AM630, may be explained by the cytotoxicity these compounds exerted on M2-10B4 fibroblast cells alone, IC_{50} values being in the range of CLL primary cells ([Table 2](#)). A dose dependent reduction of viability was also observed for PBMC of healthy individuals in suspension ([Fig 3](#)), IC_{50} values were in the same range as those of primary CLL cells under the same conditions except for RM and AM630 for which IC_{50} values were approximately twice as high ([Table 2](#)).

Cannabinoids reportedly interfere with cell-cell crosstalk which may potentially affect the therapeutic efficacy of drugs. Thus, we combined cannabinoids with fludarabine to explore potential synergies between tested drugs. Pre-incubation with cannabinoids before the addition of fludarabine generally led to a reduction in cell viability compared to cannabinoids alone ($N = 5$) ([S2 Fig](#)). These differences, however, were not statistically significant. Exception was

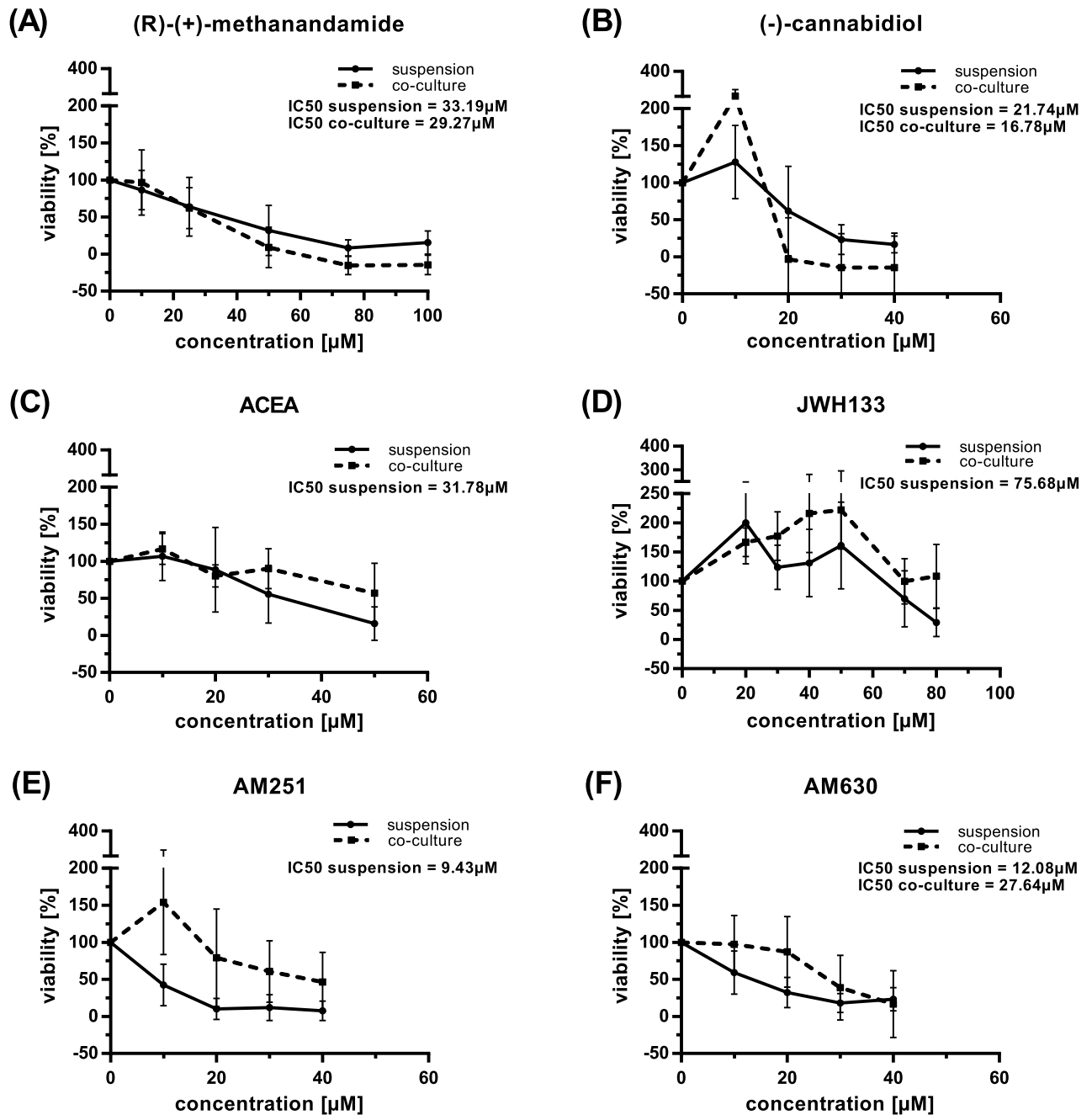


Fig 2. Cytotoxic impact of cannabinoids on CLL primary cells. PBMC from CLL patients were incubated in triplicates both in suspension culture and in co-culture with M2-10B4 mouse fibroblast cells in increasing concentrations of compounds. Viability was determined after 48h, mean values and standard deviations are shown. (A) (R)-(+)-methanandamide (N = 10). (B) (-)-cannabidiol (N = 18). (C) ACEA (N = 16). (D) JWH133 (N = 16). (E) AM251 (N = 16). (F) AM630 (N = 16). For ACEA, JWH133, and AM251, the 50% reduction in viability required for IC₅₀ calculation could not be reached in co-culture. Note different scale on x-axis in A and D.

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the CB1 agonist ACEA where the concentration dependent cytotoxicity increased reaching statistical significance in the 40 µM incubations (10 µM ACEA vs. 10 µM ACEA+Flu p = 0.117; 20 µM ACEA vs. 20 µM ACEA+Flu p = 0.076; 40 µM ACEA vs. 40 µM ACEA+Flu p = 0.047). Impact of cannabinoids and combinations were not statistically significant either when

Table 2. Impact of cannabinoids on the viability of primary cells and mouse fibroblasts.

	RM	CNB	ACEA	JWH133	AM251	AM630
CLL suspension	33.19	21.74	31.78	75.68	9.43	12.08
CLL co-culture	29.27	16.78	NR	NR	NR	27.64
HD suspension	60.13	15.09	39.01	78.12	11.44	28.51
M2-10B4	34.55	13.52	NR	NR	NR	28.27

IC₅₀ values (μM) are based on the mean values of 10 to 18 CLL PBMC and 3 HD PBMC which were incubated in triplicates in a concentration range of 0 to 100 μM of drugs for 48h. Viability of CLL cells was assessed both in suspension culture and in co-culture with the mouse fibroblast cell line M2-10B4. Healthy donor (HD) cells were incubated in suspension culture only. Abbreviations: RM, (R)-(+)-methanandamide; CNB, (-)-cannabidiol; NR, IC₅₀ not reached.

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compared to controls (N = 6) with fludarabine alone, with AMD3100 alone or with AMD3100 +Fludarabine (S2 Fig).

Serum factors may interact with cannabinoids resulting in a reduction in biologic drug activity. To investigate such an effect, primary cells were incubated with increasing concentrations of cannabinoids under reduced serum conditions, both in suspension and in co-culture (N = 5). Although not statistically significant within culture conditions, the results suggest that reduced serum concentrations added to the cytotoxic effect of the compounds, particularly at lower drug concentrations (1 and 2.5% vs. 5 and 10% FCS; S3 Fig), while this difference leveled out at higher compound concentrations. A pairwise comparison of corresponding compound concentrations between the two culture conditions (Table 3) showed a significant advantage for CLL cells in co-culture at 1 and 2.5% serum concentrations and in most cases also at 5 and 10% serum concentrations, again underlining the importance of cell-cell interaction for CLL cell survival especially at low serum conditions. Once more CNB appeared to be an exception probably due to its impact on the feeder cells (Tables 2 and 3).

Of note, sensitivity to cannabinoids was not associated with mRNA expression of either of the receptors (S4 and S5 Figs) indicating that the cytotoxic affect exerted by the tested cannabinoids was not or only partially mediated by cannabinoid receptors.

Migration assays

In T-cells, cannabinoids were shown to inhibit CXCL12 directed migration. Considering the importance of the CXCL12-CXCR4 axis in CLL, we studied whether and to which degree cannabinoids might interfere with CXCL12 mediated CLL cell migration. As shown in Fig 4, vehicle did not induce any significant changes in migration towards CXCL12, in contrast AMD3100 inhibited migration significantly (p = 0.0006) compared to controls. Both CB1&2 agonists (ACEA, JWH133) and antagonists (AM251, AM630) did not significantly influence the migratory behavior of purified CLL cells towards CXCL12. Likewise, combination of antagonist plus corresponding agonist (AM251+ACEA for CB1; AM630+JWH133 for CB2) did not lead to significant changes, either, compared to migration in vehicle containing controls. CLL cells incubated without CXCL12 showed a significantly reduced migratory behavior compared to controls (p<0.0001) (Fig 4).

Experiments with unsorted PBMC from CLL patients using the same compound concentrations but pre-incubating the cells before migration led to similar results, with significantly reduced migration in the absence of CXCL12 (p<0.0001), significant inhibition of migration towards CXCL12 in the presence of AMD3100 (p = 0.0016), and no significant block of migration in incubations with cannabinoids (S6 Fig).

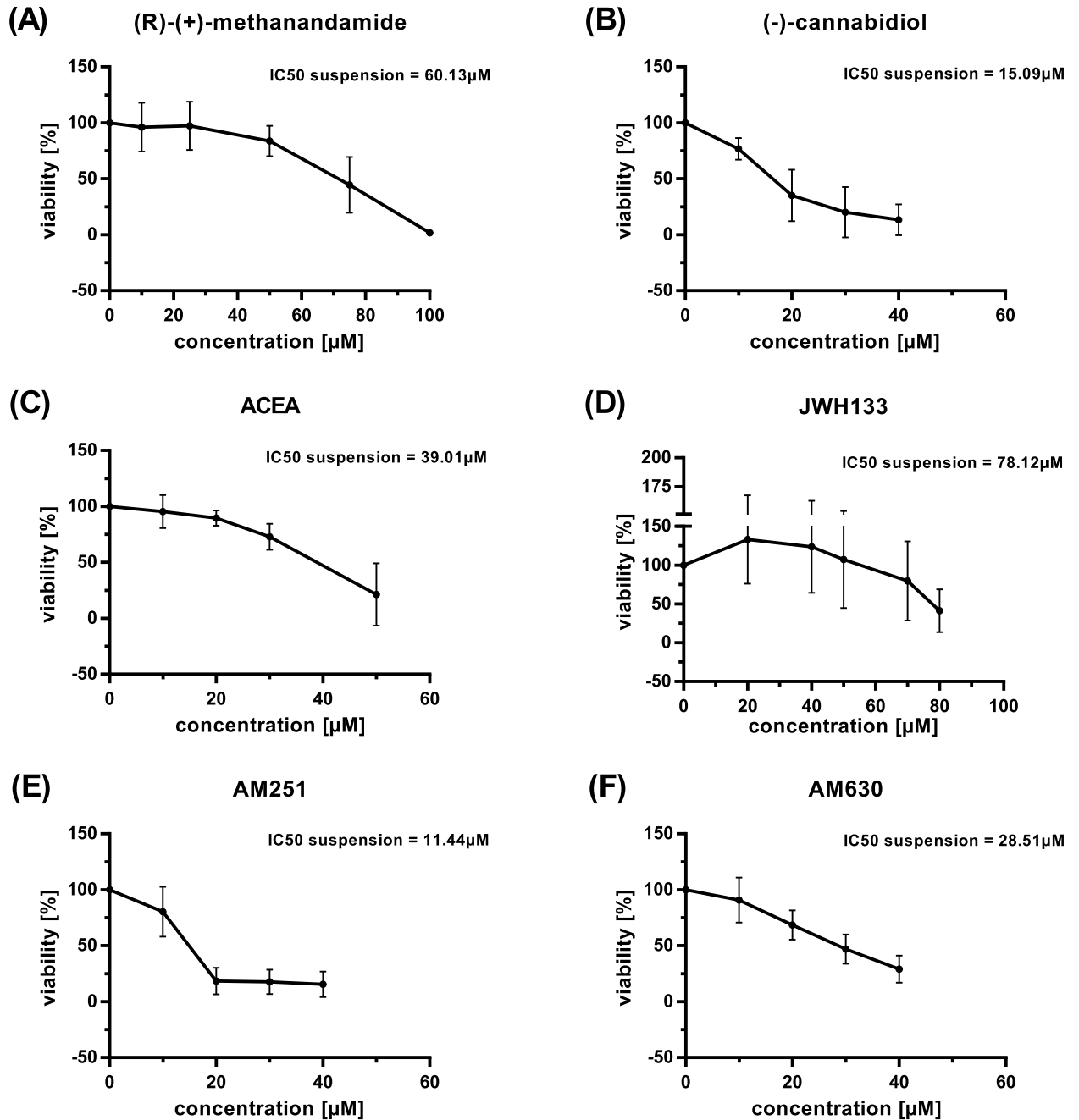


Fig 3. Cytotoxic impact of cannabinoids on primary cells from healthy individuals. PBMC from 3 healthy donors were incubated in triplicates in suspension culture in increasing concentrations of compounds. Viability was determined after 48h, mean values and standard deviations are shown. (A) (R)-(+)-methanandamide. (B) (-)-cannabidiol. (C) ACEA. (D) JWH133. (E) AM251. (F) AM630. Note different scale on x-axis in A and D, note different scale on y-axis in D.

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Discussion

Known for their psychoactive effects, cannabinoids have been used for centuries both for recreational and medical purposes. Upon the discovery of the receptors involved, drugs were developed exploiting the receptors in order to treat accompanying symptoms in various diseases [4, 64, 65]. In addition, cannabinoids displayed a number of anti-tumor effects in solid tumors

Table 3. P-values of the pairwise comparison of IC₅₀ values between suspension and co-culture in serum reduced experiments.

	RM	CNB	ACEA	JWH133	AM251	AM630
1% serum	0.0336	0.125	0.0311	0.0278	0.03	0.0278
2.5% serum	0.7254	0.03	0.0311	0.0412	0.0396	0.0468
5% serum	0.0154	0.3255	0.0279	0.0282	0.0823	0.0993
10% serum	0.05	0.2511	0.0468	0.0386	0.1021	0.0337

Student's t-test was applied, p-values reaching statistical significance or borderline significance are bold. Abbreviations: RM, (R)-(+)-methanandamide; CNB, (-)-cannabidiol;

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[4, 20, 22, 24, 26, 27, 29] and in hematologic malignancies [23, 66] which led us to determine the expression of cannabinoid receptors and to evaluate the therapeutic potential of cannabinoids in CLL.

We found that both cannabinoid receptors were overexpressed in CLL cells compared to healthy B-cells. On the mRNA level, CNR2 had a higher median expression than CNR1, which, on the other hand, had a wider range of expression compared to CNR2. When split into high and low expressing groups based on median receptor expression, only CNR1 could be shown to have prognostic value, not CNR2. Although previous publications reported an overexpression of one or both cannabinoid receptors in hematologic malignancies [22, 23, 34, 49, 67], reports regarding a potential role of this overexpression in the clinics are few [49–51]. More information is available for solid tumors, where in most studies either CB1 or CB2 expression was linked to poorer patient outcome [32, 43–48, 68]. Here, we provide for the

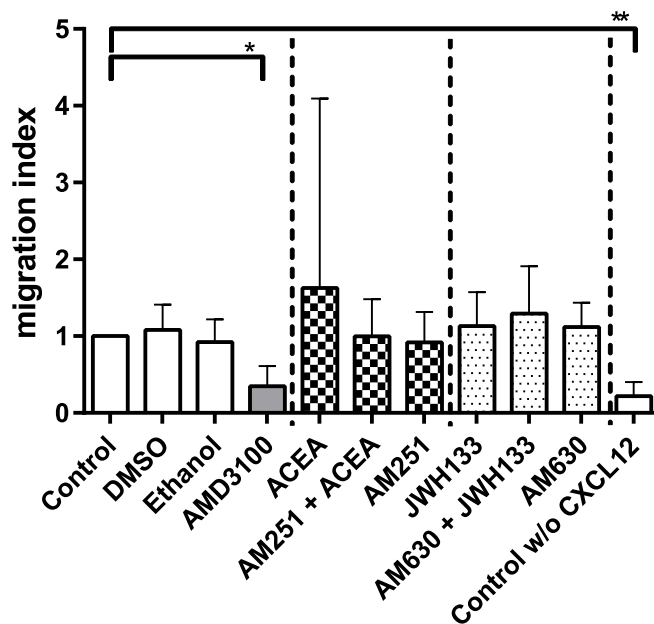


Fig 4. Impact of cannabinoids on CLL cell migration. B-cell enriched primary cells of 7 CLL patients (97.7% ± 1.04 CD19+CD5+) were incubated in transwell plates for 4h before the number of migrated cells was determined. Control experiments included CXCL12 alone (control), no CXCL12 (control w/o CXCL12), incubation with vehicle (DMSO, ethanol), and incubation with the CXCR4 inhibitor AMD3100. CLL cells were incubated either with agonist (ACEA, JWH133), antagonist (AM251, AM630), or a combination of antagonist plus agonist before migration (CB1: AMS251&ACEA; CB2: AM630&JWH133). Bars represent the mean values of migration indices + standard deviations, hatched lines indicate experimental blocks. *p = 0.0006; **p<0.0001.

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first time evidence pertaining prognostic relevance of cannabinoid receptors in lymphoid malignancies.

On the protein level, however, we could not detect any differences in expression in high and low expressing patient groups. Although some studies reported the detection of receptor protein levels in relation to mRNA expression [22, 23], we were not able to reproduce this observation. This was likely due to a lack of specificity of the antibodies used in this study. Our data are corroborated by two studies in which several commercially available antibodies were tested for CB1&2, respectively. Both groups reported great differences between the claimed specificity and the observed high degree of unspecific detection of protein by these antibodies [69, 70]. Therefore, cannabinoid receptor protein expression was excluded from further analysis.

We continued by evaluating the two receptors as potential therapeutic targets in CLL. For this, a specific agonist and antagonist pair was chosen for each receptor (CB1: agonist ACEA, antagonist AM251; CB2: agonist JWH133, antagonist/inverse agonist AM630). While these pairs are highly selective for either CB1 or CB2, respectively, a certain degree of promiscuous activity appears to be a common feature to them all. AM251 and AM630 are also ion channel activators [71], AM251 additionally acts as agonist at GPR55 and antagonist at μ -opioid receptors [40, 72]. JHW133 not only acts on CB2 but also the TRPV1 vanilloid receptor plus showed off-target effects in chemotaxis experiments in macrophages [73, 74]. Besides these two ligand pairs, two compounds were included which are being widely used in cytotoxic experiments and which have long been known for broader activity: R-(+)-methanandamide (RM) is CB1 agonist, shows activity at GPR and ion channels [1, 25], and (-)-cannabidiol (CNB) is a weak CB1 antagonist/CB2 inverse agonist interacting also with GPR55, TRPV1 vanilloid receptors and μ -opioid receptors [1, 39, 75, 76].

The dose dependent reduction in viability was more pronounced in suspension compared to co-culture. Although not statistically significant, this was reflected by different IC_{50} values under the two culture conditions and the fact that in some co-culture incubations IC_{50} values could not be calculated, in particular where the viability of M2-10B4 cells was not much impacted by the compounds. The protective effect of the microenvironment and of supporting cells in culture, particularly under treatment conditions, is known for CLL and for CLL cells [77–79].

The exceptions observed—RM, CBD, and AM630—can be explained by the toxicity these drugs exerted on the feeder cells. Likewise, healthy PBMC showed IC_{50} values similar to primary leukemic cells alone except for RM which appeared to have much less impact on healthy PBMC compared not only to the mouse fibroblasts but also to neoplastic cells, an observation that has been reported before [33]. In this line, Almestrand reported that normal PBMC subsets did not show significant changes in persons treated with rimonabant for obesity [80] while this drug induced cell death in CB1 expressing primary MCL cells in cell culture [22]. Likewise *in vitro*, rimonabant, a CB1 antagonist/inverse agonist and μ -opioid antagonist, appeared to influence healthy PBMC to a much lesser degree compared to Jurkat and U937 cells [27]. In the presented study, also AM630, the CB2 antagonist/inverse agonist, had less cytotoxic impact on healthy PBMC compared to primary CLL cells. Together this information suggests that differential or equal cytotoxic impact of cannabinoids on normal and malignant cells may be highly compound specific.

It is noteworthy, that the cytotoxic effect was not associated with either CNR1 and/or CNR2 mRNA levels. Our data differ in this respect from other studies in which the cytotoxic effect of cannabinoids observed in MCL, CLL, and HL cell lines were attributed to the overexpression of cannabinoid receptors in these cells [22, 23, 34] while other, low expressing cell lines were not inhibited [22, 23]. They also suggest that the cytotoxic effects of cannabinoids very likely are mediated by more than one receptor and one mechanism even in cases where molecules are

reportedly highly specific. As mentioned above, a certain degree of unspecific action seems to be inherent to the nature of cannabinoids, an aspect that is increasingly reflected by the literature reporting a broad range of cannabinoid activity on a variety of G-protein coupled and other receptors [1, 39–41, 66, 71–74, 81–83].

Cannabinoids supposedly interact with factors in serum which may lead to an inhibition of cellular metabolism, cannabinoid functionality, and reduction in cytotoxicity [66, 84–86]. As described previously [20, 22, 33], we found that particularly low serum concentrations enhanced the reduction in viability under both culture conditions, although less so in co-culture. These serum dependent differences, however, diminished at the highest drug concentrations where the cytotoxic effects of cannabinoids gained impact. While we do not exclude an interaction of cannabinoids with factors in serum causing an attenuation of cannabinoid cytotoxicity, we think that the impact of such effects in the course of CLL therapy is limited. In peripheral blood, under normal serum conditions, higher concentrations of cannabinoids would be required that would also lead to a high degree of toxicity in healthy cells. Under serum reduced conditions, in lymphoid organs, again higher concentrations of cannabinoids would be required since the microenvironment attenuates at least part of the cytotoxic effect of cannabinoids, and, again, healthy cells would also be influenced severely. The net effect under both conditions would be the same: a necessity of higher drug concentrations, a higher portion of healthy cells also being effected, but no real gain in killing CLL cells.

Another feature of cannabinoids should be noted. The ambivalent and variable mechanism of cannabinoid action may cause block or activation of a receptor at low concentrations while high concentrations will lead to cell death [66, 83]. Such bimodal behavior may explain the increases of viability at low concentrations in some experiments.

Interaction with the microenvironment is extremely important for CLL cells and relies to a large part on the CXCR4-CXCL12 axis, which also serves as target for therapies [79, 87]. Based on the blockage of CXCL12 induced chemotaxis and inhibition of similar receptor-ligand pairs in various lineages of peripheral blood cells [17, 88–91], we studied potential synergistic and inhibitory effects of cannabinoids with regard to the standard therapeutic agent fludarabine. Although enhanced reduction in viability could be observed in all experiments, pre-incubation with cannabinoids before addition of fludarabine did not add significantly to the reduction in viability except for the CB1 specific agonist ACEA at the highest concentration. On the other hand, our controls using the CXCR4-specific inhibitor AMD3100 did not lead to a significant synergistic effect, either. This is in contrast to a previous study where pre-treatment with AMD3100 led to a statistically significant reduction of viable CLL cells in co-culture after incubations with different drugs [79]. Although both studies used less than 10 samples for this assay (N = 8 in the Stamatopoulos study, N = 5 in this study), the difference might be attributed to the high biological variability of CLL samples and/or the different type of feeder cells used (mesenchymal stromal cells in the Stamatopoulos study, M2-10B4 mouse fibroblasts in this study) [79].

Although the impact of AMD3100 in co-incubatory experiments was limited, we found this drug to significantly block migration of CLL cells towards CXCL12. In contrast, none of our incubations with cannabinoids led to a significant inhibition of the migratory behavior of primary cells. This indicates that AMD3100 successfully interfered with the CXCR4 receptor in our experiments, but also suggests that the previously reported inhibition of the CXCR4-CXCL12 axis using cannabinoids in different blood cells [17, 89] is not valid in the CLL setting and will most likely not beneficially contribute to therapeutic regimens.

It is still unclear what role the endocannabinoid system may play in cancer and there still is much information to be gathered on how to exploit this system for anti-cancer therapy. At least for MCL some knowledge has accumulated on how cannabinoids effect malignant cells,

in addition to the possibility that the endocannabinoid system even may be involved in leukemic development [22, 24, 51, 66, 92]. Also very prominent are the studies evaluating the interference of cannabinoids with cell-cell cross-talk by different chemotaxis pairs [17, 89], which also would constitute an important aspect of targeting therapies.

We, however, could not corroborate these previous findings regarding a substantial inhibition of chemotaxis and a block of the CXCR4-CXCL12 axis by cannabinoids for CLL cells. While we did find that cannabinoids reduced viability of CLL primary cells considerably independent of CNR mRNA expression, we found healthy cells to be affected to the same degree. Thus—and in contrast to other malignancies—our data suggest cannabinoids to be of poor therapeutic potential for treatment of CLL although CNR1 mRNA expression could be determined as novel prognostic marker. Their role in CLL notwithstanding, cannabinoids may still prove useful for anti-tumor therapy in other, selected hematologic malignancies and solid tumors in which the potential of cannabinoids will have to be studied accordingly.

Supporting Information

S1 Fig. No differences in survival of CLL patients in relation to CNR2 mRNA expression.

(A) Mean overall survival (OS) for high expressing patients was 196 months vs. 230 months for low expressing patients (N = 107; p = 0.763). (B) Mean treatment free survival (TFS) in CNR2 high and low mRNA expressers was 100 months vs. 135 months in high and low expression groups, respectively (N = 107; p = 0.2290). One hundred and seven patients were included in the analysis, median mRNA expression of CNR2 (3.77) was used as cut-off. (PDF)

S2 Fig. Cytotoxic effect of cannabinoids in combination with fludarabine. CLL primary cells (N = 5) were incubated in triplicates in co-culture with M2-10B4 mouse fibroblasts and incubated for 30 minutes with increasing concentrations of cannabinoids before fludarabine (5 μ M) was added. Viability was determined after 48h. Incubations with vehicle served as control. For comparison, cells were incubated with fludarabine alone, with AMD3100 alone, and with AMD3100 in combination with fludarabine (N = 6). Mean values and standard deviations are shown. Hatched lines mark experimental blocks. The synergistic effect of the combination 40 μ M ACEA with 5 μ M fludarabine was significantly different from the effect of 40 μ M ACEA alone. *p = 0.047. Abbreviation: CNB, (-)-cannabidiol. (PDF)

S3 Fig. Cytotoxic effect of cannabinoids under serum-reduced conditions. PBMC of 5 CLL patients were incubated in triplicates for 48h in increasing compound concentrations at 1%, 2.5%, 5%, and 10% serum containing medium in suspension and in co-culture with M2-10B4 mouse fibroblasts before viability was measured. Mean values and standard deviations are shown. (A) (R)-(+)-methanandamide in suspension and (B) in co-culture. (C) (-)-cannabidiol in suspension and (D) in co-culture. (E) ACEA in suspension and (F) in co-culture. (G) JWH133 in suspension and (H) in co-culture. (I) AM251 in suspension and (J) in co-culture. (K) AM630 in suspension and (L) in co-culture. Note different scales on x- and y-axes. (PDF)

S4 Fig. Cytotoxicity of cannabinoids in relation to CNR1 mRNA expression. PBMC from CLL patients were incubated in triplicates in increasing compound concentrations in suspension and co-culture with M2-10B4 mouse fibroblast cells for 48h before viability was measured. (A) (R)-(+)-methanandamide (N = 10). (B) (-)-cannabidiol (N = 18). (C) ACEA (N = 16). (D) JWH133 (N = 16). (E) AM251 (N = 16). (F) AM630 (N = 16). The x-axis shows the measured mRNA expression for each CLL sample tested (healthy CD19 sorted cells set as 1) from highest

(left) to lowest (right) expression. Absent values may indicate that i) sample was not tested, or ii) IC_{50} could not be calculated, or iii) 50% viability reduction could not be achieved. Note different scales on Y-axis for A and D.

(PDF)

S5 Fig. Cytotoxicity of cannabinoids in relation to CNR2 mRNA expression. PBMC from CLL patients were incubated in triplicates in increasing compound concentrations in suspension and co-culture with M2-10B4 mouse fibroblast cells for 48h before viability was measured. (A) (R)-(+)-methanandamide (N = 10). (B) (-)-cannabidiol (N = 18). (C) ACEA (N = 16). (D) JWH133 (N = 16). (E) AM251 (N = 16). (F) AM630 (N = 16). The x-axis shows the measured mRNA expression for each CLL sample tested (healthy CD19 sorted cells set as 1) from highest (left) to lowest (right) expression. Absent values may indicate that i) sample was not tested, or ii) IC_{50} could not be calculated, or iii) 50% viability reduction could not be achieved. Note different scales on Y-axis for A and D.

(PDF)

S6 Fig. Impact of cannabinoids on CLL cell migration in unsorted PBMC. Primary cells of 5 CLL patients were pre-incubated with cannabinoids before being transferred to transwell plates and incubated for 4h for migration. Control experiments included CXCL12 alone (control), no CXCL12 (control w/o CXCL12), incubation with vehicle (DMSO, ethanol), and incubation with the CXCR4 inhibitor AMD3100. CLL cells were incubated either with agonist (ACEA, JWH133) or antagonist (AM251, AM630) before migration. In addition, cells were treated with antagonist before agonist incubation before migration was allowed (CB1: AMS251&ACEA; CB2: AM630&JWH133). Bars represent mean values of migration indices + standard deviations, hatched lines indicate experimental blocks.* $p = 0.0016$; ** $p < 0.0001$.

(PDF)

S1 Table. Clinical characteristics of the patients used in cannabinoid receptor mRNA analysis.

(PDF)

S2 Table. Compound concentrations and duration of incubations before initiation of migration experiments.

(PDF)

S3 Table. Comparison of patient characteristics between CNR2 high and low mRNA expressing groups.

(PDF)

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Author Contributions

Conceived and designed the experiments: KV. Performed the experiments: PF EAP TL CP. Analyzed the data: PF EAP TL CP KV. Contributed reagents/materials/analysis tools: MG PS UJ. Wrote the paper: PF EAP KV. Clinical information: MG PS UJ.

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