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ORIGINAL ARTICLE

In contrast to high CD49d, low CXCR4 expression indicates the dependency of chronic lymphocytic leukemia (CLL) cells on the microenvironment

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Abstract

CD49d and CXCR4 are key determinants of interactions between chronic lymphocytic leukemia (CLL) tumor cells and their microenvironment. In this study, we investigated the effect of CD49d and CXCR4 expressions on survival of CLL cells. Primary CLL cells were cultured with CD49d ligand, VCAM-1, or bone marrow stromal cells (BMSCs); then, apoptosis and immunophenotype analyses were performed. VCAM-1 treatment could not induce direct apoptosis protection or immunophenotype change on the CD49d-expressing CLL cells, but resulted in actin reorganization. The BMSC-induced apoptosis protection was independent from the presence of CD49d expression of CLL cells, but showed an inverse correlation with their CXCR4 expression level. We suppose that CD49d contributes to enhanced survival of leukemic cells by mediating migration to the protective microenvironment, not by direct prevention of apoptosis. Moreover, CLL cells with low CXCR4 expression represent a subpopulation that is more dependent on the microenvironmental stimuli for survival, and show increased "death by neglect" when separated from the supportive niche.

Keywords CD49d · CXCR4 · CLL · Microenvironment · Immunophenotype

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of mature $CD5^+$ $CD23^+$ B cells in the peripheral blood, bone marrow, and secondary lymphoid tissues [1]. CLL cells receive several survival signals from the tumor microenvironment, which are particularly important in CLL progression and can contribute to therapy resistance [2].

In forming supportive microenvironmental interactions, the CD49d adhesion molecule has crucial role. The CD49d is the alpha4 integrin subunit, associated with the CD29 (beta1) chain, which composes the very late antigen 4 (VLA-4; CD49d/CD29) molecule. The CD49d is one of the strongest poor prognostic factors in CLL and predicts shorter time to treatment and overall survival at diagnosis [3–6]. The CD49d/CD29 binds to fibronectin and VCAM-1, which is expressed on endothelial cells and bone marrow stromal cells (BMSCs). The involvement of CD49d in the survival of CLL cells has already been reported in the literature, but its role in direct apoptosis protection is ambiguous. $CD49d^+$ CLL cells are protected from spontaneous apoptosis due to adhesion to VCAM-1 transfected fibroblast or VCAM-1 substrate [7, 8]. Interestingly, Brachtl and their colleagues found no impact of CD49d/CD29-mediated adhesion on CLL cell viability [9].

The CD49d has an important role in leukocyte trafficking and homing, which is a crucial process in the progression of CLL [10, 11]. The CD49d/CD29 can be present in multiple activation states, which determine the binding affinity to its ligands and critical at effective homing [12]. Besides CD49d, the other key component for recirculation of CLL cells is the CXCR4 (CD184) chemokine receptor [13, 14]. CXCR4 act as the receptor of CXCL12 chemokine secreted by BMSCs and mediates chemotaxis and transendothelial migration [2, 15].

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The CXCR4 receptor is expressed at various levels on CLL cells, which is influenced by the presence of CXCL12 and BCR [15, 16]. Calissano et al. showed that CLL cells with $CD5^{high}$ $CXCR4^{dim}$ phenotype have higher proliferation activity [17], but the effect of different CXCR4 expressions on the survival of CLL cells is unclear.

Our aim was to clear the role of different CD49d and CXCR4 expressions on the survival of CLL cells by culturing them on VCAM-1-coated plates or in co-culture with BMSCs. According to our results, VCAM-1 does not directly provide survival signals through the CD49d/CD29, but resulted in F-actin formation in CLL cells with high CD49d expression. BMSCs rescued CLL cells from apoptosis independently of their CD49d expression. Furthermore, $CXCR4^{low}$ CLL cells showed higher propensity to apoptosis than $CXCR4^{high}$ cells that can be overcome by BMSCs.

These observations suggest that the CD49d has indirect impact on viability via mediating migration and adhesion to the protective lymphoid niches where tumor cells could get survival signals from other molecules; furthermore, the expression of CXCR4 can indicate the dependency of CLL cells on the signals from the microenvironment.

Materials and methods

CLL patients

Samples of 80 CLL patients (33 women and 47 men with median age of 66 [43–83]) were obtained in the study. The diagnosis of CLL was based on the World Health Organization (WHO) classification of tumors of lymphoid tissues [18]. The patients were not previously treated or had not received treatment in the last 3 years. The study was conducted in accordance with the Declaration of Helsinki and has been approved by the local Ethics Committee in Semmelweis University.

CLL cell purification and cell culture

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation over Ficoll-Histopaque (Sigma-Aldrich, St. Louis, MO, USA). The ratio of the CLL cells among the PBMCs was over 90% assessed by flow cytometry. The isolated CLL cells were cultured at a concentration of 2×10^6 cells/ml in RPMI-1640 (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), gentamycin (Sandoz, Boucherville, QC, Canada), and L-glutamine (Gibco, Carbbad, CA, USA). The CLL cells were cultured in medium alone, on VCAM-1-coated plates or in co-culture with BMSCs. The BMSC cultures, isolated from bone marrow aspirates of normal or ITP patients with no abnormal cells determined by flow cytometry, were prepared as described previously [19]. BMSCs were cultured on 24-well plates maintained at a concentration of 2×10^4 cells/ml in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 20% FBS, gentamycin, and L-glutamine. In co-culture experiments after discarding the DMEM, 2×10^6 cells/ml CLL cells per well were seeded onto the BMSC monolayer.

Coating plates and coverslips with VCAM-1

Wells of plates and plastic coverslips were coated with 10 μg/ml recombinant human VCAM-1 (R&D Systems, Minneapolis, USA) in bicarbonate buffer for 2 h at 37 °C or for 4 °C overnight. After rinsing three times with phosphate-buffered saline (PBS), non-specific binding sites were blocked with 1% bovine serum albumin (BSA) in PBS for 2 h at 37 °C. Followed by washing three times with PBS, CLL cells were seeded onto the wells of the plate or onto the coverslips.

Immunophenotype of CLL cells by flow cytometry

CLL cells were stained with fluorochrome-conjugated monoclonal antibodies summarized in Supplemental Table 1. The measurements were carried out with FACSCalibur (BD Biosciences) and analyzed by CellQuest Pro software (BD Biosciences). Twenty thousand events were acquired from every sample. The instrument settings and fluorescence compensations were regularly controlled by CaliBRITE beads; therefore, the settings are stable and the MFI values are comparable. We used isotype controls ($n = 3$) to check the aspecific binding under various culture conditions. Where positive and negative separation was needed, we gated on lymphocytes, and the double (e.g., $CD19^-/CD49d^-$)-negative population was used as an internal control. In the case of other markers, we gated on CD19-positive cells. Data were presented as geometric mean of fluorescence intensity (MFI) or in percentage of positive cells. After culturing, the immunophenotype was determined by gating on living cells. Apoptotic cells were excluded according to the lower FSC and higher SSC parameters. The cut-off value between the $CD49d^{high}$ and $CD49d^{low}$ samples was determined at 30% of positive cells [4]. The two groups with different CXCR4 expressions were based on the median value of the geometric mean fluorescence intensity of CXCR4.

Analysis of apoptosis by Annexin V/PI staining

After 3 and 7 days of culturing, CLL cells were washed twice with PBS then stained with 5 μl Annexin V-Alexa Fluor 647 (Biolegend) and propidium-iodide (PI) (1 mg/ml, Sigma-Aldrich) according to the manufacturer's instructions. Samples were measured by flow cytometry. Living cells were defined as Annexin V/PI-negative population.

Confocal microscopy

10^5 isolated CLL cells were allowed to adhere to 10 μg/ml VCAM-1 or 1% BSA (used as control)-coated coverslips for 30 min at 37 °C. After removing the non-adherent cells, the cells were fixed with 4% paraformaldehyde for 10 min at 4 °C. Then, the coverslips were washed with PBS three times, followed by permeabilizing with 0.2% Triton-X for 5 min at room temperature. Staining was performed by phalloidin-TRITC (Sigma-Aldrich) for 30 min. After washing with PBS, coverslips were placed to slides and visualized by confocal microscopy (Nikon TE 300 fluorescent microscope) then analyzed using ImageJ 1.48k program (NIH, Bethesda, MD). Because of the great difference in adhesion rate between CD49d^{high} and CD49d^{low} CLL cells, we used a "gate" by marking out the individual cells. In all samples, the same gate was used. Randomly, at least 20 cells were analyzed in every sample. In every analyzed cell, the area occupied by phalloidin-positive reaction was determined, expressed in percent. A threshold value was applied because of the background staining.

Statistical analysis

All variables were tested for normal distribution to select the appropriate parametric or non-parametric statistical procedure. Wilcoxon and Mann–Whitney *U* tests, Spearman's correlation coefficient, or by categorical variables chi-square test was used for statistical evaluation using the SPSS statistical package version 20.0 (SPSS, Chicago, IL, USA). Statistical significance was set at $p < 0.05$.

Results

VCAM-1 does not prevent the apoptosis of CLL cells

First, the anti-apoptotic effect of VCAM-1 stimulation on CD49d^{high} and CD49d^{low} CLL cells was investigated. CLL cells were cultured on VCAM-1-coated plate for 3 and 7 days and the apoptosis rate was assessed by flow cytometry. Our results show that VCAM-1 did not inhibit the spontaneous apoptosis either in CD49d^{low} or in CD49d^{high} CLL cases (percent of living CLL cells in suspension vs. VCAM-1 after 7 days: CD49d^{low} group 37.8 ± 5.6 vs. $28.7 \pm 15\%$; CD49d^{high} group 6.4 ± 1 vs. $5 \pm 3.3\%$) (Fig. 1).

To model the supportive niche of the bone marrow, we used isolated BMSCs in co-culture condition with neoplastic B cells. The viability of the CLL cells was significantly higher in co-culture with BMSCs compared to keeping them in medium only (percent of viable CLL cells in suspension vs. with stroma after 7 days: CD49d^{low} group 37.8 ± 5.6 vs. $62.5 \pm 6\%$, $p = 0.028$; CD49d^{high} group 6.4 ± 1 vs. $25.3 \pm 6\%$, $p = 0.028$). The effect of BMSCs was more prominent after 7 days than after 3 days; therefore, we used this culturing time period for further experiments. There was no difference in BMSC-induced pro-survival effect among CLL cells with different CD49d expressions. In comparison with CD49d^{low} CLL cells, the CD49d^{high} CLL cells displayed increased sensitivity to apoptosis in suspension and also in the presence of BMSCs ($p = 0.028$; $p = 0.046$) (Fig. 1).

VCAM-1 engagement induces actin polymerization in CD49d^{high} CLL cells

Although VCAM-1 stimulation on its own was not sufficient to exert anti-apoptotic effect on CLL cells, we tested the

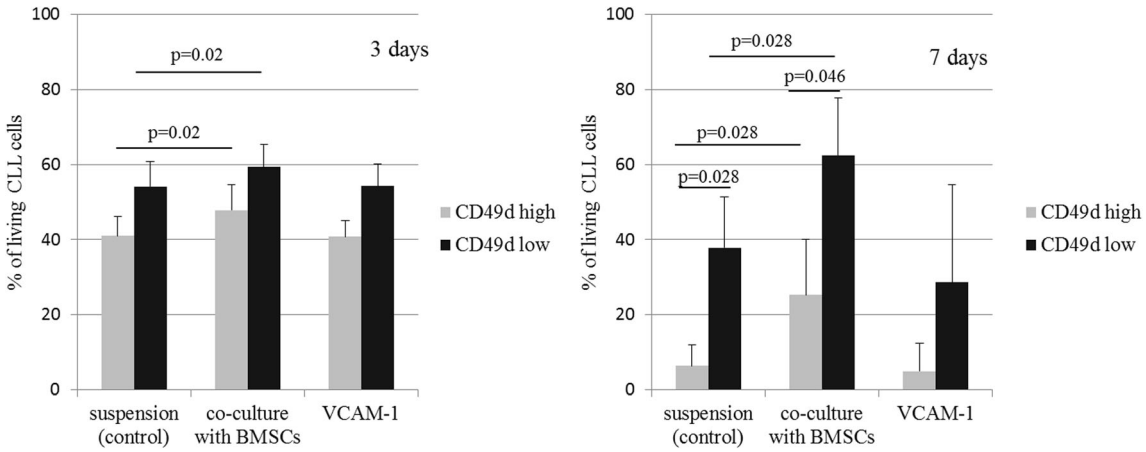

Fig. 1 CLL cell viability in BMSCs co-culture or by VCAM-1 engagement. CD49d^{high} and CD49d^{low} CLL cells were cultured in medium alone, with BMSCs or on VCAM-1 (10 μg/ml)-coated wells. After 3 and 7 days, cell viability was determined with Annexin V/PI staining, measured by flow cytometry. The bar diagrams represent the mean and SEM of 6 different cases from each CD49d group. Wilcoxon test was performed for statistical evaluation. Statistical significance was set at $p < 0.05$

adhesive capacity of the cells towards the ligand, with actin polymerization as a read-out. CLL cells with different CD49d expressions were attached to VCAM-1-coated coverslips, then were stained with phalloidin and analyzed by confocal microscopy. We observed greater adhesion rate by CD49d^{high} CLL cells (data not shown); moreover, robust F-actin formation was detected as adhered to VCAM-1-coated slides comparing with control slides (% of phalloidin-positive area on control vs. VCAM-1-coated slides 12.75 ± 0.85 vs. 27.45 ± 1.77%, $p = 0.0001$) (Fig. 2; Supplemental Fig. 1). CLL cells with CD49d^{low} phenotype did not show significant difference in actin reorganization after VCAM-1 contact (% of phalloidin positive area on control vs. VCAM-1 coated slides 11.35 ± 0.79 vs. 12.01 ± 0.37%).

In contrast to VCAM-1, BMSCs modulate the immunophenotype of CLL cells

Interaction with microenvironmental elements can change the immunophenotype of CLL cells [19, 20]. We examined the immunophenotype alteration of CD49d^{high} and CD49d^{low} CLL cells after VCAM-1 treatment or in BMSCs co-culture. Markers involved in migration, activation, proliferation, and co-stimulation (expression of CD49d, CD5, CD38, CD19, CXCR4, CD126 (IL-6R α), CD80, and CD86) were analyzed before and after 7 days of culturing in medium alone, on VCAM-1 substrate, or together with BMSCs. VCAM-1 stimulation alone did not change the immunophenotype of CLL cells either in CD49d^{high} or in CD49d^{low} cohort (data not shown). In co-culture conditions with BMSCs, CLL cells showed significantly elevated levels of CD5, CD49d, CD19, and CD126 compared to CLL cells cultured in medium alone (mean MFI of CD5 expression 158.5 ± 24.3 vs. 114.4 ± 17.8, $p = 0.002$; of CD49d expression 93 ± 21.4 vs. 62.7 ± 15, $p = 0.041$; of CD19 expression 779.1 ± 95.8 vs. 531.4 ± 48.1, $p = 0.002$; of CD126 expression: 33.8 ± 4.2 vs. 30.4 ± 4.1, $p = 0.020$) (Fig. 3). Compared the two groups with different CD49d levels, greater increase in CD5 expression was observed after stromal cell contact by CD49d^{high} CLL cells ($p = 0.03$). Regarding the other surface markers, we did not find any correlation with the CD49d level. BMSCs co-culture did not result in significant changes in the expression of CD38, CD80, and CD86 in our culture condition. Furthermore, CXCR4 expression was decreased on CLL cells in co-culture after 7 days (mean MFI of CXCR4 expression 124.1 ± 31.1 vs. 189.6 ± 40.7, $p = 0.01$). We checked the aspecific binding under various conditions by using isotype controls. We did not find significant changes in the fluorescence intensity of isotype controls in samples cultured in medium or co-culturing with BMSCs.

CLL cells with low CXCR4 expression require greater stroma-support, but show enhanced apoptosis rates

Analyzing the level of CXCR4 and CD49d positivity (and CD49d MFI as well) ($n = 80$), an inverse correlation was observed ($p = 0.001$) (Fig. 4a, Supplemental Fig. 2). Culturing CLL cells with different CXCR4 expressions for 7 days in medium, CXCR4^{low} CLL cells showed greater rate of apoptosis compared with CXCR4^{high} CLL samples ($p = 0.03$) (Fig. 4b). BMSCs increased the survival of CLL cells in both CXCR4 group (percent of living cells after 7 days in

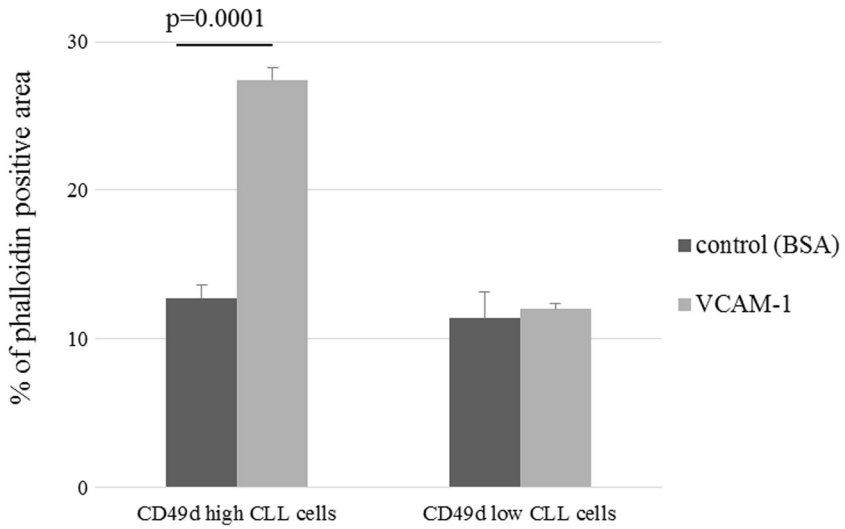

Fig. 2 Actin-polymerization of CD49d^{high} and CD49d^{low} CLL cells upon VCAM-1 stimulation. Primary CD49d^{high} or CD49d^{low} CLL cells were allowed to adhere to VCAM-1- or BSA-coated control coverslips for 30 min at 37 °C. After washing off the non-adherent cells with PBS, the cells were fixed and permeabilized, then stained with phalloidin-TRITC. Cells were visualized by confocal microscopy. As result, the area occupied by phalloidin-positive reaction was given. Displayed results are the mean and SEM of phalloidin-positive area by 3 different samples. Wilcoxon test was performed for statistical evaluation. Statistical significance was set at $p < 0.05$

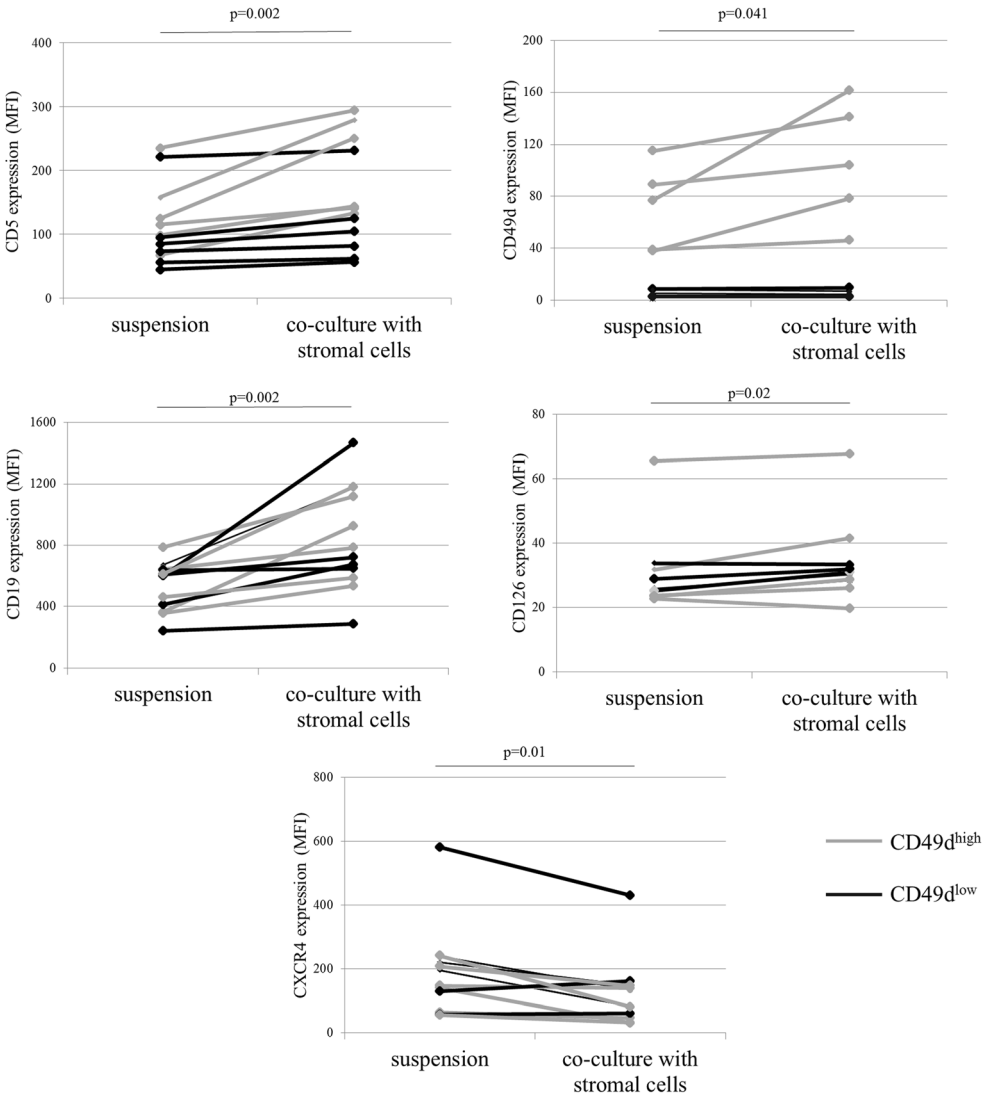

Fig. 3 The surface molecule expression of CD49d^{high} and CD49d^{low} CLL cells in BMSC co-culture. Isolated CLL cells from 12 different CLL blood samples (6 from each CD49d group) were cultured with BMSCs or in medium alone (suspension-control) for 7 days; then, the expression of CD5, CD49d, CD19, CD126, and CXCR4 was analyzed by flow cytometry gated on living cells. The CD49d^{high} cases were displayed with gray; the CD49d^{low} cases with black lines. Wilcoxon tests were performed for statistical evaluation. Statistical significance was set at $p < 0.05$

suspension vs. in co-culture condition in CXCR4^{low} group 11.1 ± 4.6 vs. 39.4 ± 8.7%, $p = 0.018$; in CXCR4^{high} group 43.6 ± 9.9 vs. 51.8 ± 9.8%, $p = 0.042$) (Fig. 4b). The CXCR4^{low} CLL cells were more susceptible to apoptosis both in medium and on BMSCs, but higher proportion of cells was rescued in the co-culture with stromal cells compared with CXCR4^{high} cells (Fig. 4b). The increased survival of CXCR4^{low} CLL cells in the co-culture reveals a higher dependence on BMSCs.

CXCR4 does not, but CD49d predicts poor prognosis

We have next investigated whether the microenvironmental sensitivity is related to clinical parameters. It is well studied that CD49d^{high} CLL patients show advanced disease stage, LDH level at diagnosis, and shorter time to treatment (TTT), which was confirmed by our clinical data (Table 1) [3, 4, 21]. Interestingly, we observed lower absolute lymphocyte count (ALC) at diagnosis by the CD49d^{high} cases, but it did not reach statistical significance (64.1 ± 12.6 vs. 77.6 ± 18 g/l).

Regarding the CXCR4 expression of CLL patients, no significant difference was detected in any clinical parameters between the CXCR4^{low} and CXCR4^{high} cohorts (Table 1). Patients with Binet A stage represent a highly heterogeneous disease group. Analyzing the Binet A CLL cases according to the CXCR4 expression, significantly elevated LDH level was found in the CXCR4^{low} patient group (351.6 ± 39.2 vs. 206.4 ± 21.8; $p = 0.003$).

Discussion

The main aim of this study was to analyze the effect of CD49d and CXCR4 expressions on the survival and immunophenotype of CLL cells engaged by VCAM-1 or bred under conditions that mimic the bone marrow microenvironment.

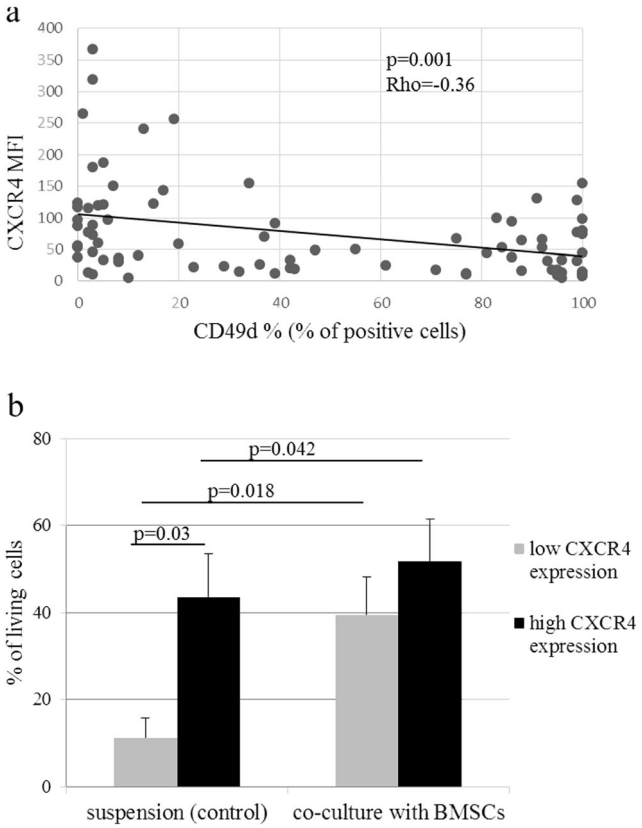

Fig. 4 The correlation of CXCR4 level with CD49d positivity and the spontaneous apoptosis rate of CLL cells with different CXCR4 expressions. **a** Correlation between the CXCR4 MFI and CD49d positivity (expressed in %) on CLL cells. The expressions of CXCR4 and CD49d have been measured on freshly isolated CLL cells by flow cytometry ($n = 80$). Correlation was evaluated with the Spearman correlation coefficient. Significance was determined at $p < 0.05$. **b** CLL cells with low CXCR4 level take greater advantage of BMSC-protective effect after culturing for 7 days, though showed increased cell death. CLL cells with low or high CXCR4 expression were cultured in medium alone or in the presence of BMSCs. After 7 days, cell viability was determined with Annexin V/PI staining, measured by flow cytometry. The bar diagrams represent the mean and SEM of 6 different cases from each groups. Wilcoxon tests were performed for statistical evaluation. Statistical significance was set at $p < 0.05$

The survival effect of CD49d has already been investigated in CLL, but its role is proved to be controversial. We found that the CD49d does not mediate survival signals by VCAM-1 stimulation. Furthermore, we detected that the anti-apoptotic effect of BMSCs on CLL cells was independent of the CD49d expression, although VCAM-1 is expressed on the stroma surface [19]. Our findings are consistent with the results of Brachtl et al. who used CD49d blocking antibody, which disrupts the VCAM-1 binding but it did not alter the survival rates of CLL cells [9]. In contrast, in the work of Zucchetto et al., CLL cells were cultured with murine L-fibroblast transfected with human VCAM-1 or VCAM-1 substrate alone and found apoptosis protection, but this phenomenon was observed after 7 days and was more prominent after 21 days [7, 8]. Because of the big spontaneous apoptosis rates in our experiments, we cultured the CLL cells at the longest for 7 days. The different culturing conditions might be an explanation for the different results.

We studied whether the missing anti-apoptotic effect is the result of impaired VLA-4 activation upon inside-out activation, e.g., by chemokine. However, we detected that at unstimulated conditions, the CD49d/CD29 complex is expressed in inactive, low-affinity confirmation on the surface of CLL cells, but undergoes the normal activation process after stimulation, e.g., VCAM-1 (Supplemental Fig. 3). Our data are in agreement with previously published results, which reported that the CD49d/CD29 is not in pre-activated form on CLL cells [22].

We, however, did not detect any direct role of CD49d-VCAM-1 binding in the survival of CLL cells. Our data shows robust actin-remodeling in CD49d^{high} CLL cells upon VCAM-1 binding as was shown by Buchner et al. [23]. Therefore, CD49d may be important in several processes associated with cytoskeleton-remodeling, such as migration and adhesion. More studies indicate higher homing rate to bone marrow [9, 22, 24] and enhanced motility on VCAM-1 by CD49d^{high} CLL cells [25]. Additionally, our results show elevated CD49d level in the BMSCs co-culture, which mimic the tissue microenvironment, similarly to other studies [19, 20]. CLL cells in the lymph node also have upregulated CD49d expression [26]. Analyzing our clinical data, CD49d^{high} CLL patients have lower ALC at diagnosis, but it did not reach statistical significance. The higher peripheral lymphocyte count can be explained by the greater migration and retention in the lymphoid tissues by the CD49d-positive CLL cells.

We confirmed that the ratio of CD49d-positive cells and the level of CXCR4 show inverse correlation, similarly as described by Ganghammer et al. [22]. High CD49d and weak CXCR4 expressions are associated with clinical lymphadenopathy [27, 28]. We detected higher spontaneous apoptosis rates by CD49d^{high} CXCR4^{low} CLL cells compared to CD49d^{low} CXCR4^{high} CLL cells. CLL cells are in continual recirculation between the peripheral blood and lymphoid organs. Malignant B cells, which encounter the tissue-derived anti-apoptotic and growth signals in the microenvironment, may be more susceptible to the lack of the favoring stimuli. We suppose that this "death by neglect" effect can be resulted in the observed big spontaneous apoptosis rate among CD49d^{high} CXCR4^{low} CLL cells, which might represent a recently emigrated population from the beneficial lymphoid niches. Furthermore, our co-culture experiments showed increased survival of CXCR4^{low} CLL cells, indicating this population is more dependent on the microenvironmental stimuli. The CXCR4 expression may reflect the microenvironmental dependency, but it does not have prognostic impact at diagnosis according to our data, although in the heterogeneous Binet

Table 1 Clinical characteristics of CLL patients regarding the different CD49d and CXCR4 expressions

	$CD49d^{high}$	$CD49d^{low}$	Significance (CD49d cohorts)	$CXCR4^{low}$	$CXCR4^{high}$
Rai stage			$p = 0.018$		
Early stage (Rai 0, I, II)	18/27	25/27		26/35	16/18
Advanced stage (Rai III, IV)	9/27	2/27		9/35	2/18
Binet stage			$p = 0.017$		
Binet A	11/26	17/26		19/36	9/17
Binet B	7/26	7/26		8/36	7/17
Binet C	8/26	2/26		9/36	1/17
LDH (U/l)	394.9 ± 37.7	315.1 ± 32.2	$p = 0.013$	367.1 ± 32	334.6 ± 41
TTT (days)	642.7 ± 158.5	1248.4 ± 276	$p = 0.016$	827.9 ± 192	1176.4 ± 298
ALC (g/l)	64.1 ± 12.6	77.6 ± 18	n.s.	76.7 ± 15	54.4 ± 14.2

LDH lactate dehydrogenase, *TTT* time to treatment, *ALC* absolute lymphocyte count, *n.s.* non-significant

A patient group low CXCR4 expression was significantly associated with elevated LDH level at diagnosis, which may indicate high turnover rate of CLL cells. However, this can be a result of the inverse correlation with CD49d expression, which is reported to predict progressive disease in Binet A CLL [21].

The characteristics of CLL cells are modulated by microenvironmental triggers contributing to enhanced survival and proliferation. As our findings, VCAM-1 alone was not sufficient to induce phenotypical changes, but BMSCs co-culture resulted in higher CD49d, CD19, CD5, and CD126 and diminished CXCR4 level as was shown in partly in our previous work [29]. BMSC-derived antigens can stimulate BCR on CLL cells [30], which may give rise to the increased CD19 and CD5 levels and the downregulation of CXCR4 [31]. However, the diminished CXCR4 level could be also the effect of the CXCL12 produced by BMSCs. The higher CD5 expression may indicate the activation of malignant B cells, which was more prominent in the $CD49d^{high}$ population. CLL cells express higher level of CD126 compared to normal B cells, which is correlated with STAT-3 expression and chemoresistance [32]. In our study, the CD126 level was increased on CLL cells in the presence of BMSCs, which indicate its role in neoplastic B cell-tumor microenvironment cross-talk and the microenvironment-induced therapy resistance.

We showed in this study that VCAM-1 engagement by the CD49d/CD29 did not prevent CLL cells from apoptosis or induce immunophenotypical changes, but resulted in actin remodeling. We suppose that the CD49d/CD29 complex may contribute to enhanced survival of CLL cells by mediating migration and adhesion to the protective microenvironment, not by direct inhibition of apoptosis. Furthermore, CD49d-positive CLL cells express lower level of CXCR4 and represent a population being more dependent and sensitive to the microenvironmental stimuli. These results suggest to study and consider the expression of CXCR4 on CLL cells which can predict the effect of a microenvironment-targeted therapy. On the basis of our data, it may be reasonable to test targeted therapies whether they have the capacity to mobilize $CD49d^{high}/CXCR4^{low}$ cells from the protective microenvironment making them very susceptible to apoptosis in these poor prognostic cases.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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