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ORIGINAL ARTICLE

Different proliferative and survival capacity of CLL-cells in a newly established *in vitro* model for pseudofollicles

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Chronic lymphocytic leukemia (CLL) is a malignancy of mature B-lymphocytes that manifests in a variety of clinical courses. The accumulation of CLL-cells is primarily caused by defective apoptosis; however, a higher proliferative capacity has also been found to correlate with poorer prognostic factors. Proliferating CLL-cells are confined to specialized structures called pseudofollicles, which contain CLL-cells, T-lymphocytes, and stromal cells. We established an *in vitro* model for pseudofollicles to characterize the behavior of CLL-cells in relation to clinical courses with different outcomes. Only CLL-cells from progressive clinical cases were inducible to proliferate by a combination of soluble CD40L/IL-2/IL-10 in co-culture with stromal cells. Proliferating CLL-cells showed a higher and more extensive expression of antigens, which are important in T–B-cell interactions such as CD40, MHC II, and adhesion molecules. IL-4 increased interferon regulatory factor-4 expression and induced a specific immunophenotype, which may imply plasmacytic differentiation. Furthermore, it was shown that co-cultured stromal cells protected CLL-cells from apoptosis. CLL-cells from clinically indolent cases had a far worse survival rate in medium than the cells from poor prognostic cases. Thus, we can assume that not only a different resistance to apoptosis, but also proliferation contributes to the progression of CLL resulting in bone marrow failure with thrombocytopenia and anemia.
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Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of monoclonal, resting B-lymphocytes expressing a typical pattern of antigens.[1]

Despite the similar cytology and immunophenotype, CLL manifests itself in a variety of different clinical courses. The disease burden based on clinical staging systems (e.g. RAI stage III: anemia, or IV: thrombocytopenia) is the most significant prognostic factor for survival.[2] The poor prognostic impact of the germ line immunoglobulin heavy chain variable region (IgV_H) gene,[2] zeta-chain-associated protein kinase (ZAP-70) expression,[3] and CD38 expression[4] were confirmed at an early stage of the disease.

The progressive increase in lymphocyte counts despite low mitotic rates of dividing cells led to the concept that CLL is primarily attributable to defective apoptosis.[5] A proliferating pool, however, has been identified in so-called proliferation centers or pseudo-follicles (PC), composed of cycling CLL-cells (Ki-67$^+$, Survivin$^+$, p27$^-$, Bcl-2$^+$, CD23^{hi}), delicate stromal cell networks and activated (CD40L$^+$) T_H-cells.[6–9] The transcription factor interferon regulatory factor-4 (IRF4 [multiple myeloma oncogen 1(MUM1)]) can also be differentially expressed in proliferation centers compared with the surrounding lymphocytes.[10]

Evidences from a number of *in vitro* experiments with CD40L and T-cell-derived cytokines indicate the importance of T-lymphocytes in CLL but conflicting results were published concerning CD40 and IL-4 with regard to CLL-cell proliferation-induction and anti-apoptotic effects.[7,11–15] CD40 activation combined with IL-2 and IL-10 was found to synergize in proliferation induction in CLL.[16] IL-4 normally induces IgE class switching and additional IL-10 enhances IgE production by promoting *in vitro* plasma cell differentiation.[17]

Recent experiments revealed a different proliferative capacity in CLL: *in vivo* measurements showed that a higher birth rate of CLL-cells correlates with disease progression.[18] Unmethylated CpG motif containing oligodeoxynucleotide (CpG-ODN) stimulated CLL-cells of patients with a worse prognosis through Akt-mTOR (mammalian target of rapamycin) pathway to proliferate more often *in vitro*.[19] The higher LDH level found in CLL with 'poor prognostic cytogenetics' as described by Jahrsdörfer *et al*.[20] also refers to the significance of CLL-cell neogenesis in disease progression.

Despite an obvious long half-life *in vivo*, CLL-cells die fast *in vitro* during a short-term culture in medium.[21] Co-cultures either with human bone marrow-derived stromal cells (BMSCs) or even with mouse fibroblasts significantly reduced the percentage of apoptotic CLL-cells.[13,22]

The aim of this study was to establish an *in vitro* cell culture system similar to proliferation centers to investigate the differences in the behavior of CLL-cells in relation to prognostic and clinical features. Our experiments indicate that only CLL-cells from progressive cases usually presenting with thrombocytopenia were able to proliferate on T-cell-derived cytokine stimulation in co-culture with BMSCs. We could show that CLL-cells had different survival capacities in medium with a correlation to prognostic factors. Furthermore, T-cell-derived cytokines exerted characteristic effects on the immunophenotype

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and gene expression of CLL-cells. These data confirm the concept of the heterogeneity of CLLs and the role of the microenvironment in the pathophysiology of this disease.

Materials and methods

Patients

Recruitment of 21 CLL patients, 7 women and 14 men, with median age of 65 (39–84) years was approved by the ethics committee of the University of Regensburg. All participants gave written informed consent. The diagnoses were based on standard clinical immunophenotyping and cytology. The patients were either untreated or had not been treated in the previous 3 months. Table 1 summarizes the main clinical data of the patients, whereas the therapy states of the treated patients are listed in Supplementary Table 4.

Preparation of BM mesenchymal stromal cell (BMSC) cultures

Ten different bone marrow aspirates, taken for routine diagnostic procedures, were applied for BMSC cultures. Specimens were chosen exclusively from untreated lymphoma or ITP patients without detectable abnormal cells. Therefore, we always applied allogenous BMSCs. Primary human BMSC cultures were established as described earlier,[22] and details are shown in the Supplementary information at Leukemia's website.

Homogeneity of BMSCs was proved by the investigation of adhesion molecule expressions (ICAM1, VCAM1) and cytokine secretions (IL-6, IL-8, SDF-1) (data not shown).

Separation of CLL-cells from peripheral blood

Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque technique with Biocoll (Biochrom, Berlin, Germany). For separation of CLL-cells, surface staining of the isolated peripheral blood mononuclear cells was performed with four monoclonal antibodies: fluorescein isothiocyanate-labeled anti-glycophorin A, phycoerytrin-labeled anti-CD14, anti-CD56, and allophycocyanin-labeled anti-CD3. The unstained cells in the lymphoid gate were sorted by a FACSAria cell sorter (BD Biosciences, Heidelberg, Germany). The prepared cell populations contained usually >98% CLL-cells, assessed by direct immunofluorescence staining using a FACSCanto flow cytometer (BD Biosciences). Source and specificity of the applied monoclonal antibodies are listed in Supplementary Table 1, shown in the Supplementary information at Leukemia's website. Staining specificity was verified by respective isotype controls (BD Biosciences).

Culture conditions

10^6 purified CLL-cells/0.5 ml/well were cultured in DMEM (PAN Biotech GmbH, Aidenbach, Germany) supplemented with 10% FCS. The following co-culture/culture conditions were applied: (1) BMSCs; (2) BMSCs and soluble CD40 ligand (sCD40L); (3) BMSCs, sCD40L, and IL-4; (4) BMSCs, sCD40L, IL-2, and IL-10; (5) only medium; (6) medium and sCD40L; (7) medium, sCD40L, and IL-4; and (8) medium, sCD40L, IL-2, and IL-10. The CLL-cells were harvested after 84 h and further analyzed without sorting again.

The applied BMSCs were entirely developed and showed no morphological sign of development into macrophages or fat cells during the co-culture (data not shown).

The human recombinant reagents listed above were used as follows: CD40L: 1 μg/ml (PeproTech EC, Hamburg, Germany or R&D Systems, Wiesbaden-Nordenstadt, Germany), IL-4: 10 ng/ml, IL-2: 100 U/ml, IL-10: 10 ng/ml (all from PeproTech EC).

Multiparameter flow cytometric DNA analysis

First, surface antigens were stained with fluorochrome-conjugated monoclonal antibodies according to the manufacturers'

Table 1 Clinical and prognostic characteristics of the studied patients

Patient	*P[a]/NP[b]*	*PLT[c], $\times 10^9$/l*	*HGB[d]*, g/100ml	*Lymphocyte count, $\times 10^3/\mu l$*	*ZAP70 +, %*	*CD38 +, %*	*Mutation state*	*Mutation ratio, %*	*V-gene*	*Age*	*Sex*	*Previous chemotherapy*	*Bone marrow infiltration*
20	P	25	6.6	9.4	72	31.2	UM[e]	0	1–69	66	M	Yes	>90%
9	P	49	10.1	42.0	70	50.0	UM	0	1–69	73	M	Yes	60%
28	P	100	13.7	43.0	53	44.8	UM	1.8	3–23	67	M	No	90%
11	P	114	12.4	87.0	41	38.0	UM	0	3–48	57	M	No	na
23	P	115	11.6	119.0	35	1.6	M[f]	12.4	3–7	39	F	No	na
29	P	115	9.6	63.2	41	2.5	M	4	3–30	78	F	Yes	na
33	P	140	11.3	65.0	54	32.0	UM	0	3–23	84	M	No	na
17	P	166	11.5	304.0	83	10.0	UM	0.4	1–2	57	M	Yes	60%
13	NP	140	12.9	69.3	6	8.0	ND[g]	ND	ND	79	F	No	na
16	NP	150	12.2	42.6	21	45.0	UM	0	3–33	72	F	Yes	77%
19	NP	153	14.3	16.0	37	8.7	UM	0.4	3–30	58	F	Yes	80%
21	NP	167	14.5	15.0	32	0.9	UM	0	1–69	55	M	No	60%
31	NP	180	13.0	74.0	13	6.6	UM	0	1–69	66	M	No	na
27	NP	211	15.7	115.7	11	4.1	M	8.0	4–34	64	M	No	na
30	NP	166	14.1	8.0	8	0.5	M	9.6	1–18	78	M	No	na
18	NP	171	17.0	20.0	11	4.6	M	9.1	3–30	67	M	No	20%
15	NP	185	15.0	15.7	35	5.0	M	7.0	1–69	68	M	No	20%
12	NP	200	14.3	40.0	5	18.0	M	5.9	2–5	73	M	No	na
22	NP	207	14.9	42.8	6	0.3	M	4.5	4–61	46	M	No	na
32	NP	220	14.4	10.0	13	2.0	ND	ND	4–55P	76	F	No	na
14	NP	223	13.9	38.0	6	10.0	M	5.1	3–53	73	F	No	na

Notes: [a]*P*, cells with inducible proliferation *in vitro*; [b]NP, cells without inducible proliferation *in vitro*; [c]PLT, count of platelets; [d]HGB, hemoglobin concentration; [e]UM, unmutated; [f]M, mutated; [g]ND, not determinable; na, not available at the time of the experimental setup.

data sheets. The applied monoclonal antibodies are listed in Supplementary Table 1. Red blood cells were lysed and lymphoid cells were fixed simultaneously by FACS lysing solution containing 1.5% formaldehyde (BD Biosciences). After washing, the cells were resuspended in 2% DAPI solution (Molecular Probes, Eugene, OR) and analyzed on a LSRII flow cytometer (BD Biosciences). Data acquisition and analysis were performed using either FACSDiva software (BD Biosciences) or Wincycle software for cell-cycle analysis (Phoenix Flow Systems, San Diego, CA).

Antigen expression and cell-cycle distribution were analyzed within the population of CLL-cells (CD19/CD5 double positive). Antigen density was expressed in relative fluorescence intensity. See details in the Supplementary information at Leukemia's website.

Flow cytometric analysis of apoptosis

Approximately 5×10^5 sorted or harvested CLL-cells were washed in cold PBS and resuspended in 100 µl of annexin-binding buffer (BD Biosciences) containing 1 µg/ml propidium iodide (PI) (Sigma Aldrich, Taufkirchen, Germany) and 5 µl of fluorescein isothiocyanate-conjugated annexin V (BD Biosciences). After 15 min incubation period, 400 µl of annexin-binding buffer were added and the cells were analyzed on a LSRII flow cytometer using FACSDiva software. Viable cells were PI- and annexin V-negative.

The detection of DNA fragmentation was performed by DAPI staining as described above. Apoptotic cells contain less DNA than living cells and can be detected in the sub-G1 area.

Calculation of cell survival in vitro

Cultured CLL-cells were counted in a Casy Cell Counter Model DT (electronic pulse area analysis) (innovatis AG CASY Technology, Reutlingen, Germany) after 84 h *in vitro*. The living population was corrected by subtracting the annexin V-positive fraction from the absolute cell count. We set the number of preculture live (annexin V-negative) cells to 100%. The ratio between the corrected living cell number before and after co-culture resulted in the surviving rate of the CLL-cells under different culture conditions.

Immunohistochemical staining of IRF4

Immunohistochemistry for IRF4 was performed on formalin-fixed CLL-cells of cytospin specimens from 12 co-cultured CLL-cases according to the manufacturers' data sheets. See details in the Supplementary information at Leukemia's website.

Immunofixation

Hydragel (Sebia Inc., Norcross, GA) high-resolution immunofixation technique was applied to detect immunoglobulins and kappa or lambda free light chains in the culture supernatant. Immunofixation was carried out with undiluted medium according to the manufacturer's recommendations. Serum from a plasmocytoma patient and polyclonal serum were used as positive controls.

Gene expression analysis

For gene expression analysis, mRNA was isolated using QIAAmp mRNA isolation mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Firststrand cDNA synthesis of a total of 2 µg RNA was performed using ImProm-II reverse transcriptase system (Promega, Mannheim, Germany) according to the supplier's recommendations. Gene expression analyses with TaqMAN gene expression assays (AssayOnDemand, Applied Biosystems, Darmstadt, Germany) were carried out in a HT-7900 real time PCR machine (Applied Biosystems) in 384 well plates. Cycling conditions matched the specifications designed for AssayOnDemand probes by Applied Biosystems. mRNA expression was calculated using the delta–delta C(t) method of qRT-PCR and quantity is given in arbitrary units compared with the 18 s gene as housekeeper in all cases.

V_H Family assignment

To determine the V_H gene family used by the CLL-cells, genomic DNA was amplified and sequenced. Detailed information about the applied methods are available as Supplementary information at Leukemia's website.

Statistical analysis

Statistical analysis was performed with SPSS, version 16.0. Graphs denote box and whisker plots. Comparisons of groups of data were performed using the two-tailed *t*-test and statistical significance is considered with *P*-values $\leqslant 0.05$.

Results

CLL-cells change their immunophenotype under the influence of BMSCs, whereas soluble CD40L exerts only a marginal effect on antigen expression

CLL-cells up-regulated the majority of the investigated antigens by the end of the 84 h co-culture with BMSCs. The expression of activation antigens such as CD23, CD69, co-receptors HLA-DR, B7-2 (CD86), and adhesion molecules such as ICAM1 (CD54) and CD49d were increased three- to four-fold, whereas the expression of CD19, CD40, and P-glycoprotein-1 (Pgp-1, hyaluronate receptor, CD44) increased only 1.5–2-fold (Figure 1a). The expression of CD45 did not change.

To further characterize the differentiation capacity of co-cultured CLL-cells, several genes involved in plasma cell maturation, such as IRF4, a known target for IL-4 and CD40L in normal B-cells,[23] B-cell lineage-specific activator protein coded by paired box domain 5 (PAX5) critical for B-cell commitment,[24] B-lymphocyte-induced maturation protein-1 (BLIMP1) [PR domain containing 1 (PRDM1)], a transcriptional repressor of PAX5,[25] and Syndecan-1 (SDC1/CD138), a marker for plasma cells,[26] were selected for gene expression analysis.

CLL-cells from peripheral blood strongly expressed PAX5 (Figure 2a) and IRF4 mRNA (Figure 2b), whereas the BLIMP1 expression was very weak (Figure 2c). CD138 mRNA was either undetectable or expressed at a very low level in the CLL-cells of the peripheral blood as well as in the cultured CLL-cells (data not shown).

CLL-cells reduced the expression of both PAX5 and BLIMP1 on BMSCs (Figures 2a and c). IRF4 mRNA levels increased in most of the cultures with BMSCs, but these changes were not significant (Figure 2b).

In general, soluble CD40L (sCD40L) slightly altered the immunophenotype (Figure 1b) and gene expression (Figure 2) of sorted CLL-cells induced in the co-culture with BMSCs. sCD40L significantly enhanced the expression intensities of antigens like CD11c, CD18, CD40, CD54, and HLA-DR and decreased the expression of CD20 and CD184; however, the range of

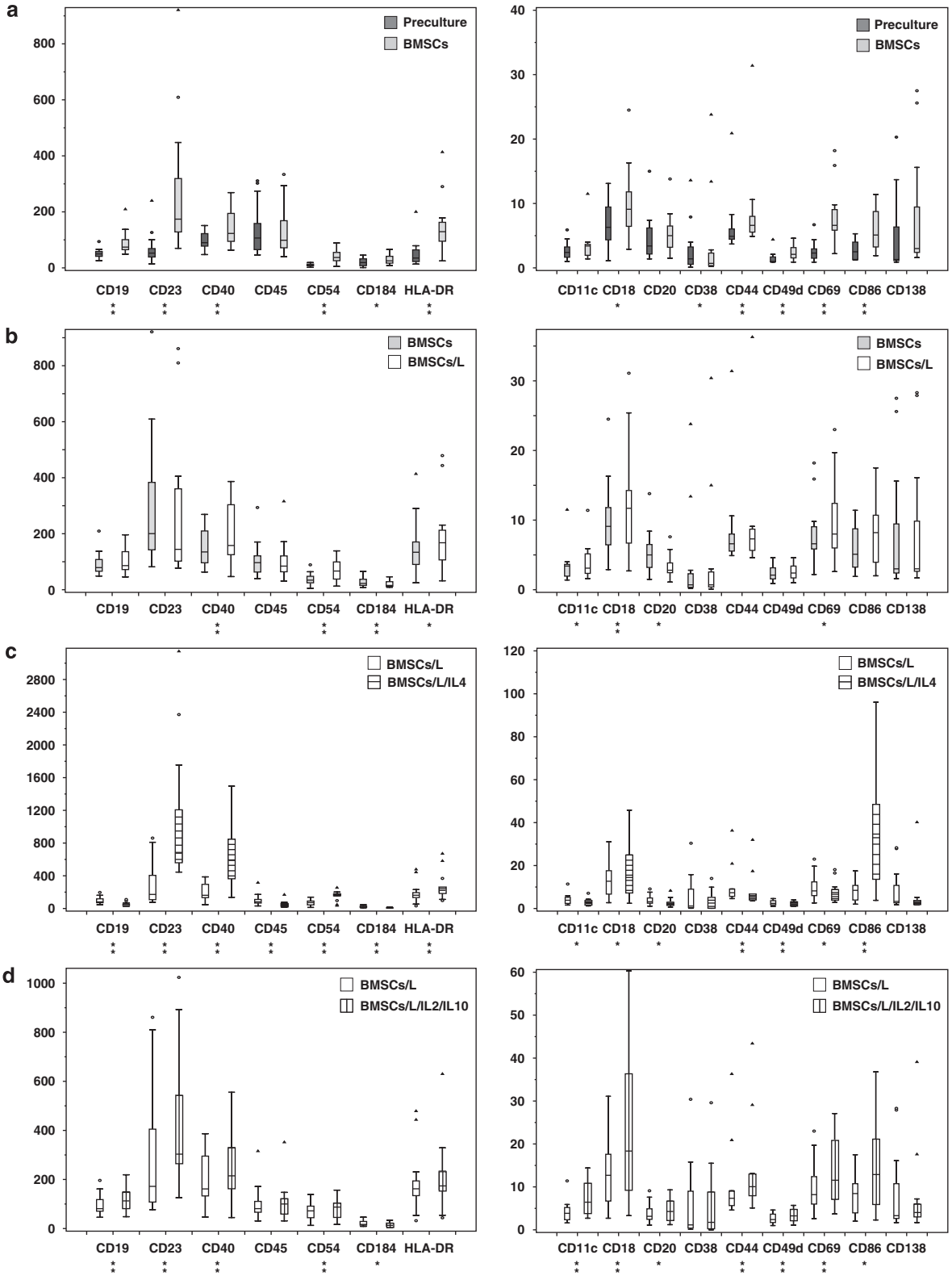

Figure 1 BMSCs and T-cell-derived cytokines (IL-4 and IL-2/IL-10) have marked effects on the immunophenotype of CLL-cells. 10^6 sorted CLL-cells per well were cultured on BMSCs with/out different combinations of cytokines; soluble CD40L (1 μg/ml), or sCD40L and IL-4 (10 ng/ml) or sCD40L and IL-2 (100 U/ml) + IL-10 (10 ng/ml). The cells were harvested after 84 h and immunophenotyped. (**a**) Dark gray boxes: antigen expression before co-culture; black boxes: antigen expression after co-culture with BMSCs. (**b**) Light gray boxes: antigen expression after co-culture with BMSCs, white boxes: antigen expression after co-culture with BMSCs and sCD40L. (**c**) White boxes: antigen expression after co-culture with BMSCs/sCD40L, fasciated boxes: antigen expression after co-culture with BMSCs/sCD40L and IL-4. (**d**) White boxes: antigen expression after co-culture with BMSCs/sCD40L, lengthwise striped boxes: antigen expression after co-culture with BMSCs/sCD40L and IL-2/IL-10. Relative fluorescence intensities (RFI) of 15–18 independent experiments are depicted as box and whisker plots. Rings indicate outliers and triangles indicate extreme outliers. Significance (two-tailed) is calculated with the paired *t*-test with $*P \leqslant 0.05$, $**P \leqslant 0.001$.

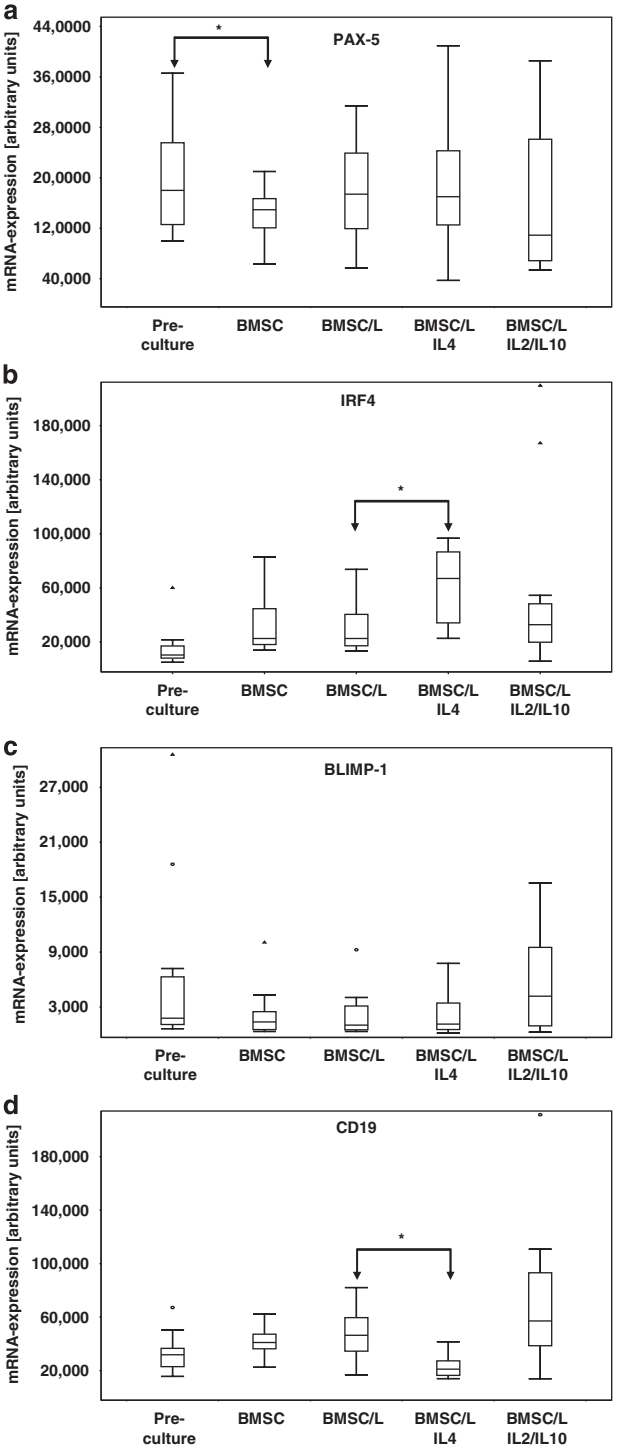

Figure 2 Transcription analysis of CLL-cells. mRNA from CLL-cells cultured under different conditions was isolated and analyzed for the expression of PAX-5 (**a**), IRF4 (**b**), BLIMP-1 (**c**), and CD19 (**d**) by TaqMAN gene expression assays. mRNA was quantified as relative mRNA level and given in arbitrary units relative to 18 s RNA expression in each case. The contamination by BMSC-mRNA was ascertained with ~0.1% by measuring VCAM-1 expression and was graded as negligible. Box 1: sorted, peripheral blood CLL-cells, box 2: CLL-cells on BMSCs, box 3: CLL-cells on BMSCs/sCD40L, box 4: CLL-cells on BMSCs/sCD40L and IL-4, box 5: CLL-cells on BMSCs/sCD40L and IL-2/IL-10. Relative mRNA expression levels of 11–12 independent experiments are depicted as box and whisker plots. Rings indicate outliers and triangles indicate extreme outliers. Significance (two-tailed) is calculated with the paired *t*-test with $*P \leqslant 0.05$, $**P \leqslant 0.001$.

alterations was smaller compared with those induced by BMSCs (Figures 1a and b).

CLL-cells preserved their differentiation capacity in response to IL-4

IL-4 stimulation of CLL-cells cultured on BMSCs with sCD40L dramatically up-regulated several antigens including CD23, CD40, CD54, and CD86. IL-4 also induced the expression of HLA-DR and CD18, but decreased CD45, the activation antigen CD69, the B-cell specific antigens CD19 and CD20, the adhesion antigens CD11c, CD44, and CD49d, and the chemokine receptor CD184 (CXCR4). IL-4 did not influence the expression of CD38 and CD138 (Figure 1c). On the mRNA level, CD19 was also down-regulated by IL-4 (Figure 2d).

In 10 of 12 investigated cases, IL-4 in combination with sCD40L and BMSCs significantly increased the expression of IRF4 mRNA compared with BMSCs with sCD40L (Figure 2b).

To confirm the transcription analysis, immunohistochemistry was performed for IRF4 expression in trephine biopsy sections and on cytospins of cultured CLL-cells. IRF4 positive cells were found in all investigated biopsy sections either forming centers or being scattered among the surrounding lymphocytes (Figure 3a). The sorted CLL-cells expressed IRF4 antigen heterogeneously (Figure 2b). In co-culture with BMSCs (Figure 3c), BMSCs/sCD40L (Figure 3d), or BMSCs/sCD40L/IL-2/IL-10 (Figure 3g), IRF4 was obviously not induced. IL-4 exerted a visible effect on IRF4 expression, increasing the number of strong positive cells and inducing mild expression in the majority of cells in all investigated specimens (Figure 3e). Interestingly, the larger cells contained a higher level of IRF4 with strong positivity in their nuclei (Figure 3f).

Expression of PAX5 mRNA (Figure 2a), critical for B-cell characteristics, was not altered by IL-4 similar to plasma cell specific genes such as BLIMP1 (Figure 2c) and CD138 (data not shown). IL-4 induced the expression of IRF4 and decreased CD19, CD20, and CD45, which may indicate plasma cell differentiation, although BLIMP1 and CD138 were not induced. To examine this possibility, the immunoglobulin secretion of CLL-cells was investigated by immunofixation, but none was detected in the supernatant of co-cultures in a series of five experiments (data not shown).

IL-2 in combination with IL-10 exerted strong alterations in the BMSCs/sCD40L-induced CLL-cell immunophenotype

IL-2 and IL-10 induced the majority of the investigated adhesion molecules including CD11c, CD18, CD44, CD49d, and CD54, whereas the SDF1 receptor CD184 decreased. The B-cell specific antigens CD19 and CD20, the activation markers CD23 and CD69, and costimulators for T-cells, like CD40 and CD86 were also induced. IL-2 and IL-10, however, did not change the expression of HLA-DR and CD45, but influenced CD38 heterogeneously among cases (Figure 1d). The extent of up-regulation of CD23, CD40, CD86 by IL-2 and IL-10 was much lower than by IL-4 (Figures 1c and d). The pattern and extent of up-regulation of these antigens were similar in all cases, and were independent of the proliferation (Supplementary Figure 1 on Leukemia's website).

On the mRNA level, the expression of PAX5 was nearly maintained, whereas CD19, IRF4, and BLIMP1 were up-regulated by sCD40L in combination with IL-2 and IL-10 in co-cultured CLL-cells compared with sCD40L alone, but did not reach significance (Figure 2).

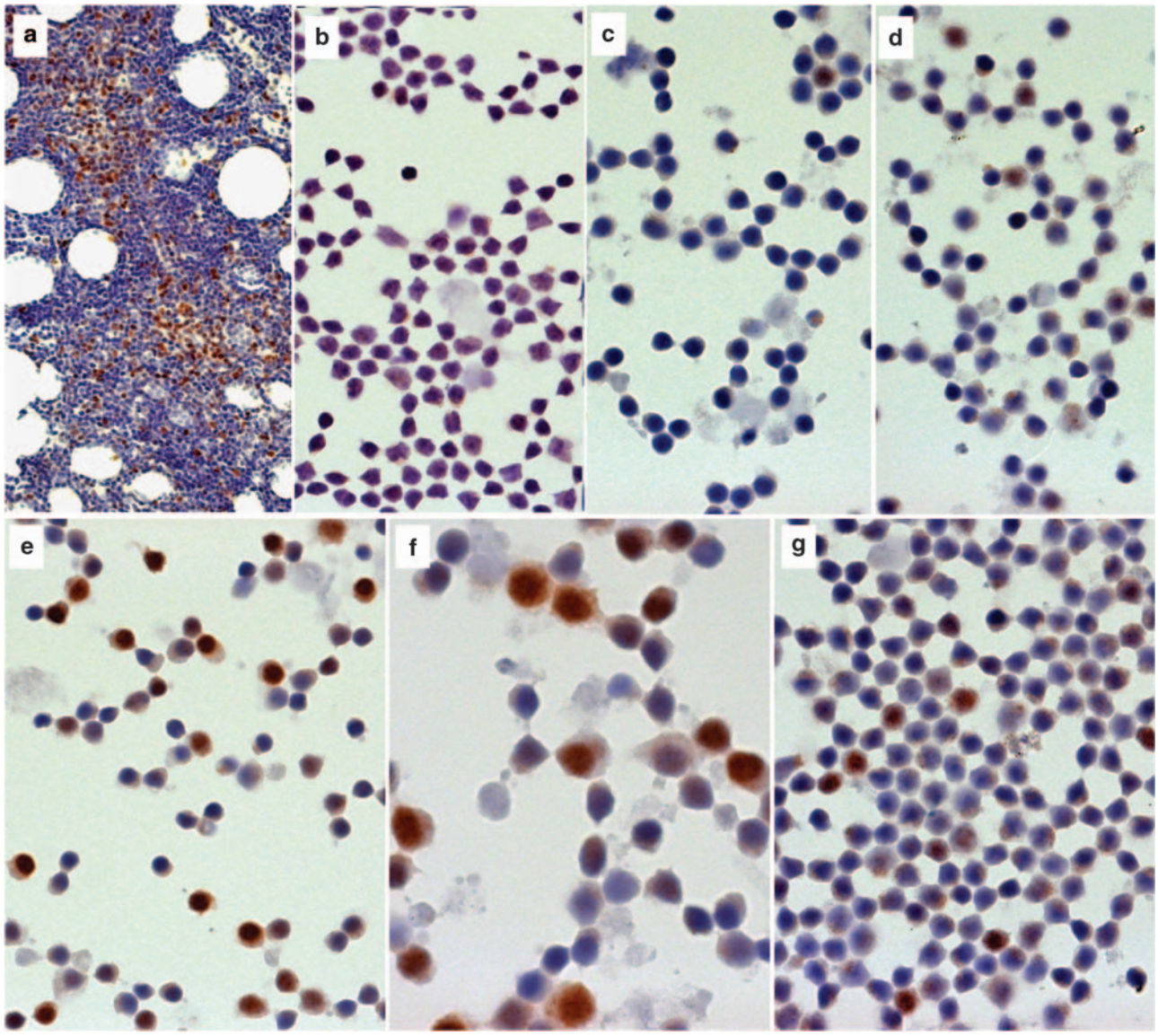

Figure 3 IRF4 expression in CLL-cells. (**a**) IRF4 immunohistochemistry of a trephine biopsy from a CLL case with IRF4-positive (brown) cells, counterstained with hematoxylin (original magnification $\times 20$). (**b**–**g**) IRF4 immunohistochemistry of cytospin specimens of sorted and cultured CLL-cells (original magnification $\times 40$, **f**: $\times 100$): (**b**) sorted CLL-cells, (**c**) CLL-cells on BMSCs, (**d**) CLL-cells on BMSCs and sCD40L, (**e**) and (**f**) CLL-cells on BMSCs/sCD40L and IL-4, (**g**) CLL-cells on BMSCs/CD40L and IL-2/ IL-10. Microscope Imager Z1, camera AxioCam MRC, acquisition software Axiovision Rel 4.6.3, objective lenses: EC Plan Neofluar $20 \times /0.5$, EC Plan Neofluar $40 \times /1.3$ oil, EC Plan Neofluar $100 \times /1.3$ oil (Zeiss), adobe photoshop for image processing.

CLL-cells from clinically progressive cases were able to proliferate

The applied staining method with mild fixation and high DNA accessible DNA dye DAPI hardly influenced the antigen expression levels and resulted in low spectral overlaps and small CVs of DNA histograms fulfilling the requirements for accurate multiparameter DNA analysis.[27] No dividing CLL-cells were detectable in the peripheral blood of the 21 cases studied (Figure 4a). Neither BMSCs, sCD40L, and IL-4 on their own, nor their combination induced CLL-cell division in our cultures (data not shown). To make sure that the missing proliferative effect of the soluble CD40L is not due to an insufficient concentration or a lack of functionality of the synthetic molecules, CLL-cells were stimulated with sCD40L from two different manufacturers in multiple concentrations (0.5, 1.0, 3.0, and 5.0 μg/ml). However, we did not detect any proliferation regardless of the concentration or origin of sCD40L (data not shown).

In 8 of our 21 cases (Table 1), we could induce cell division of CLL-cells co-cultured with BMSCs and stimulated by a combination of sCD40L, IL-2, and IL-10 (Figure 4a).

Interestingly, CLL-cells from all thrombocytopenic cases were able to proliferate. We could also induce cell division in two cases with platelet counts around the minimum normal level ($140–166 \times 10^9$/l). None of the patients had isolated thrombocytopenia without other signs of disease progression indicating autoimmune thrombocytopenia. In all, 6 of 8 patients with proliferating CLL-cells had an unmutated IgV_H region, and 7 of these 8 patients were also anemic, all of them expressed ZAP-70, but 3 of them carried hardly any CD38 antigen (Table 1). The proportion of cells in S and G2/M phase varied between 0.7 and 7.8% at the end of the co-culture period (Figure 4b). The

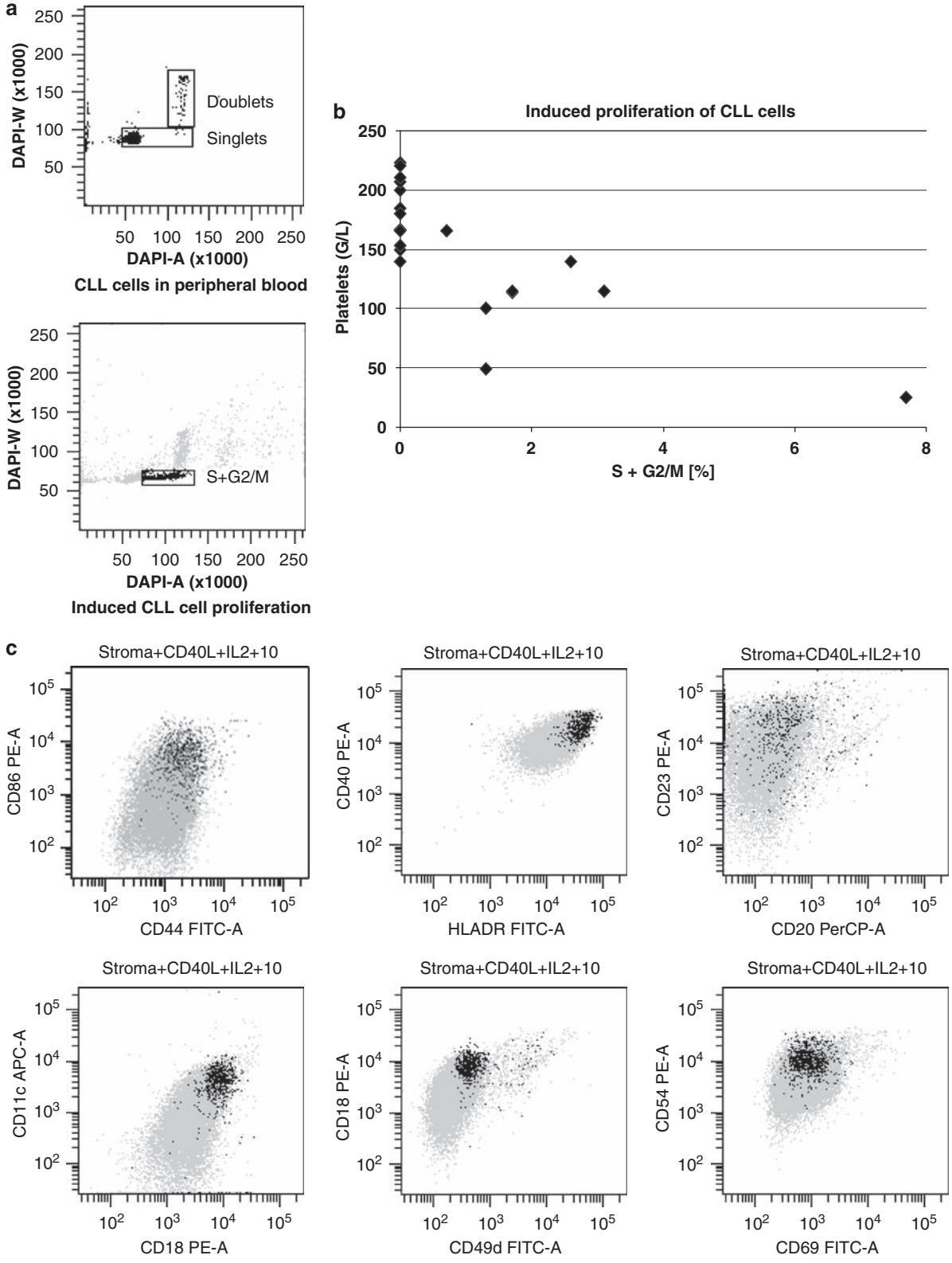

Figure 4 Proliferation induction in CLL-cells. (**a**) Cell-cycle analysis of CLL-cells ($CD19^+$, $CD5^+$) by DAPI staining: top: CLL-cells from peripheral blood showing no proliferation (no cells in S or G2/M phase); bottom: CLL-cells after 84 h of co-culture with BMSCs/sCD40L and IL-2/IL-10 showing induced proliferation with cells in S or G2/M phase. (**b**) The diagram displays the association between thrombocytopenia and induced proliferation of CLL-cells. The range of S + G2/M phase fraction varied between 0.7 and 7.8% at the end of the co-culture period. (**c**) The proliferation-associated immunophenotype is shown after gating S + G2/M phase cells (black events) and G0/G1 phase cells (gray events).

different proliferative responses were not associated with proliferation of contaminating T cells, because the ratio of T-lymphocytes remained under 1% at the end of the co-culture (data not shown). Also, differences in the expression of IL-2Rα (CD25) were not responsible for different proliferation capacities as the range of up-regulation varied widely on IL-2 and IL-10

and did not correlate with proliferative responses (Supplementary Figure 1 on Leukemia's website).

The knowledge of a specific proliferation-associated surface immunophenotype in CLL would be helpful to find small proliferating populations especially in bone marrow aspirations and to perform a reliable DNA analysis. To determine the specific antigen expression pattern of the dividing cell pool, we gated the S and G2/M phase fraction. Adhesion antigens such as CD44, CD54, and the integrins CD18, CD11c, and CD49d were expressed by the proliferating cells at a high level. Furthermore, co-stimulators for T-cell activation like CD40, HLA-DR, and B7-2 (CD86) were also intensively expressed. The majority of proliferating cells carried the low affinity IgE receptor CD23 brighter than the nonproliferating cells (Figure 4c), but, interestingly, CD69, another activation antigen, was expressed with the same intensity as the majority of non-cycling cells. By backgating to FSC/SSC plot and pulse processing, we could ensure that individually higher antigen expressions on proliferating cells cannot solely be attributed to differences in cell size. Unfortunately, the DAPI-gated S-G2/M phase and G0–G1 phase CLL-cells could not be resolved by the expression of the investigated surface antigens measured by flow cytometric analysis as their fluorescence intensities were found in the range of all cells including G0–G1 phase CLL-cells. Therefore, it was not possible to gate the cycling population by means of fluorescence intensities of these antigens (Figure 4c).

CLL-cells have different survival capabilities in medium

Confirming previous apoptosis studies on CLL, we could show that BMSCs protected CLL-cells from spontaneous apoptosis *in vitro*, whereas the majority of CLL-cells died when cultured in FCS-supplemented medium demonstrated by the larger degree of DNA cleavage ('sub-G_1' region) (Figure 5a). BMSCs did not provide complete protection from apoptosis as the proportion of living (annexin V-negative) CLL-cells dropped to 40–60% on average (Figure 5b) during co-culture. In medium, however, a different survival rate was observed in our sample cohort. CLL-cells, able to proliferate (P), showed far better survival in medium than the cells from non-proliferating cases by the end of the 84 h incubation period (Figures 5b–d).

One group of non-proliferating cases (NP/1) with unmutated IgV_H (Pat 16, 19, 21, 31) and/or peripheral blood lymphocytosis above $5 \times 10^4/\mu l$ (Pat 13, 27) also showed higher survival rates in medium (similar to the proliferating cases) than cells from indolent cases (NP/2) with mutated IgV_H and lower lymphocyte

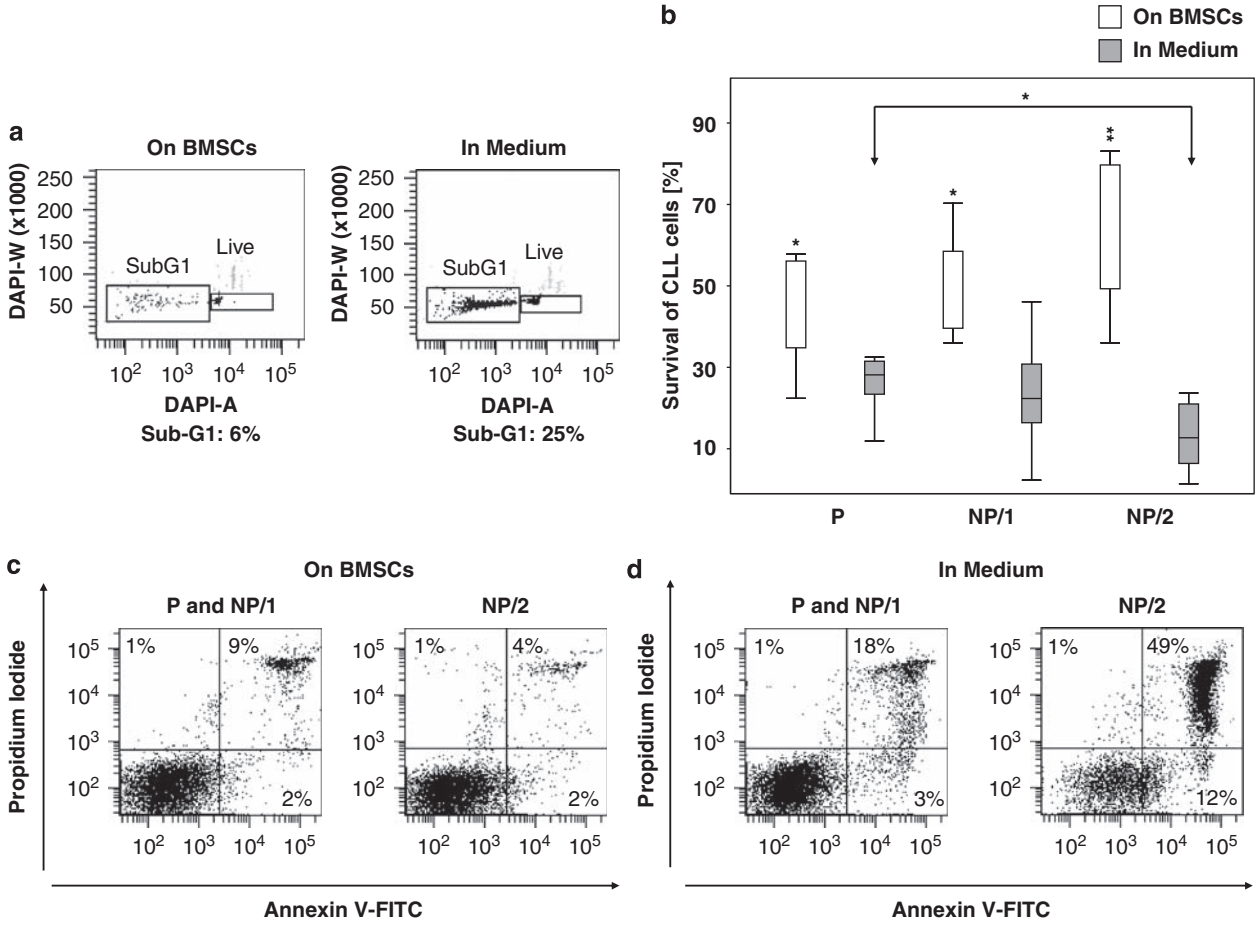

Figure 5 Survival of CLL-cells on BMSCs and in medium. (**a**) Measurement of cellular DNA content of fixed CLL-cells by DAPI staining. Fixed, apoptotic cells lost their degraded DNA resulting in a reduced DNA content (sub-G_1 region) (**b**) At the end of the 84 h culture period, cell survival was calculated on the basis of absolute cell counts and annexin V-staining. P: cases with inducible proliferation, NP/1: non-proliferating cases with poor prognosis, NP/2: non-proliferating cases with better clinical prognosis. White boxes: survival of CLL-cells on BMSCs, gray boxes: survival of CLL-cells in medium. Results for the survival proportion (%) of P, NP/1, and NP/2 of 8, 6, and 7 independent experiments, respectively, are depicted as box and whisker plots. Rings indicate outliers and triangles indicate extreme outliers. Significance (two-tailed) is calculated with the paired and impaired *t*-test with $*P \leqslant 0.05$, $**P \leqslant 0.001$. (**c**–**d**) Annexin V and PI staining of CLL-cells in the presence of BMSCs or in medium alone. Viable cells are double negative, early apoptotic cells are annexin V-positive and PI negative, late apoptotic cells are double positive.

counts (Pat 12, 14, 15, 18, 22, 30, 32) (Figures 5b–d). Interestingly, CLL-cells from indolent cases benefited more from BMSCs than proliferating cases, although this difference was not statistically significant (Figure 5b).

Soluble CD40L alone, in combination with IL-4 or with IL-2 and IL-10 exerted very heterogeneous, differential effects on the survival of CLL-cells both on BMSCs and in medium (data for IL-2 and IL-10 are shown in Supplementary Figure 2 on Leukemia's website).

Discussion

In this report, we introduce an *in vitro* model for proliferation centers in CLL composed exclusively of primary human cells (BMSCs and sorted CLL-cells) with/out T-lymphocytic cytokines (sCD40L, IL-2, IL-10, IL-4) to learn more about the mutual interactions between these components and find reasons for the variable clinical courses of CLL. The commercially available human BMSC lines (e.g. HS-5) immortalized by a lymphotropic virus did not seem to be ideal for cultivating lymphocytes; therefore, human BMSCs isolated from several healthy individuals and human recombinant soluble CD40L were combined in this culture system.

The immunophenotyping of CLL-cells co-cultured with BMSCs reflects a strong activation that may enhance the adhesion of CLL-cells to BMSCs and improve communication between CLL-cells and T cells.

In normal B cells, simultaneous engagement of IL-4R and CD40 leads to high levels of IRF4 expression through STAT6 and NFκB signaling pathways.[23] We demonstrated that these signaling pathways are probably preserved in CLL-cells because IRF4, and its known targets CD23 and HLA-DR, were strongly up-regulated by IL-4. Neither sCD40L nor IL-2 combined with IL-10 influenced significantly the expression of IRF4; therefore, we assume a possible key role for IL-4 in the induction of IRF4 expression in CLL. Besides HLA-DR, the other IL-4 up-regulated co-receptors B7-2 (CD86) and CD40 may additionally serve for the interaction of CLL-cells and T-lymphocytes *in vivo*.

The transition from B-cell to plasma cell is coordinated by the elevated expression of IRF4 resulting in an up-regulation of BLIMP1.[28] The plasma cell differentiation process is accompanied by the appearance of CD138 after the plasmablastic stage and with the decrease in the surface antigens CD19, CD20, and CD45.[26] The up-regulation of IRF4 and the down-regulation of surface CD19, CD20, and CD45 on CLL-cells by IL-4 in our model may imply the onset of plasma cell differentiation. This differentiation, however, seems to be incomplete as the PAX5 expression was maintained, and the zinc-finger transcription factor BLIMP1, as well as the typical surface antigens CD38 and CD138, were not induced in CLL-cells. High CD23 expression indicated the lack of BLIMP1 in IL-4-stimulated CLL-cells, because BLIMP1 represses the CD23b promoter by physically interacting with IRF4.[23] Several causes could be responsible for the incomplete plasmacytic maturation of CLL-cells on IL-4 stimulation in this cell-culture system: the lack of IL-10 stimulation, the permanent sCD40L induction, the short incubation period[17,29,30] and a possible maturation arrest, being a general feature of neoplastic cells.[31] A similar CXCR4 down-regulating effect of IL-4 was recently shown on human monocytes.[32] Lower CXCR4 expression was identified on lymph node-infiltrating CLL-cells compared with peripheral blood CLL-cells,[33] which may reflect an *in vivo* IL-4 effect besides the CXCL12-induced receptor endocytosis.[34]

Bone marrow stromal cells express IL-4R[35] and on IL-4 stimulation they up-regulate VCAM1,[36] therefore IL-4 may also exert an indirect effect on CLL-cells through BMSCs. Despite this fact, the observed changes in immunophenotype and mRNA expression are most likely due to a direct effect of IL-4, because some of them were also shown on leukocytes without BMSCs.[15,23,32]

Combined IL-2 and IL-10 stimulation induced also a specific pattern of antigens on CLL-cells: an increased expression of integrins, hyaluronate receptor (CD44), activation antigens and co-receptors. The role of integrins and CD44 is undisputable in CLL: CD18 and CD49d mediate the adhesion to BMSCs.[22] CD44 or β-integrins expressing CLLs show diffuse bone marrow infiltrations or require more frequent therapy.[37]

The preceding data clearly demonstrate that proliferating CLL-cells in the peripheral blood, if they exist at all, are under the sensitivity level of multiparameter flow cytometric DNA analysis. In contrast to previous reports,[7,11] soluble trimeric CD40L alone did not induce proliferation in our model system, although different concentrations of two different sCD40L molecules were applied. We showed that the proliferation inducing stimulus (IL-2 and IL-10) pushes just a portion of CLL-cells into proliferation, similar to the effect of CpG-ODN.[19] The proliferating cases belonged to the poor prognostic group. Of interest, a low platelet count indicated most sensitively the ability of CLL-cells to proliferate. We assume that the proliferation of CLL-cells leads to stronger bone marrow infiltration resulting in lower platelet counts and anemia. All available trephine bone biopsies from the investigated cases displayed diffuse bone marrow infiltration in support of this theory. The low rate of induced proliferation (S and G2/M phase cell fractions fewer than 4% in 7 of 8 cases) was probably due to the lack of CLL- and T-cell contact.[38] Further studies are required to determine whether proliferative response changes with disease progression.

The measurement of the proliferating pool in CLL would be reasonable, because the increase in proliferating fractions correlates with the biological progression of non-Hodgkin lymphomas.[39] In CLL-infiltrated bone marrow, the proportion of the dividing population is usually very low; therefore, the performance of a reliable DNA analysis is not only technically demanding, but almost impossible. Unfortunately, we did not manage to identify any proliferation specific surface immunophenotype to simplify the gating of the proliferating pool. The stronger expression of adhesion antigens and co-receptors may reveal the capability of the proliferating pool to better interact with T-lymphocytes. The brighter CD23 expression on cycling cells can underscore the authenticity of this *in vitro* cell culture, as this phenomenon was also described on the larger Ki-67 positive cells within the proliferation centers.[8] We suppose that the observed immunophenotype changes are the result of regulation rather than selection of low antigen expressing cells because the proliferating cells express a batch of antigens higher (e.g. CD40, HLADR, CD18) and others lower (e.g. CD69, CD184) than the resting cells.

Defective apoptosis is postulated to be the major factor responsible for the *in vivo* accumulation of CLL-cells[5]; therefore, the survival of CLL-cells was also measured in the present work. Our results also confirmed the beneficial effect of BMSCs on the survival of CLL-cells.[22] The slightly better survival of the indolent cells on BMSCs might be due to the maintenance of higher Bcl-2 expression described in CLL-cells with 'good cytogenetics' as suggested by Jahrsdörfer *et al*.[20] Interestingly, survival of CLL-cells varied widely in medium: more cells from each CLL case with inducible proliferation and from those non-

proliferating cases having germline IgV_H or higher peripheral blood lymphocytosis (P or NP/1) were able to survive in medium. The recently described higher Bcl-2-associated X protein (Bax) degradation activity can explain the lower *in vitro* apoptosis of poor prognostic CLL cases.[40] In contrast to previous reports, neither CD40 activation alone nor in combination with IL-4 significantly improved cell survival *in vitro* regardless of the presence of BMSCs.[12,13,41] This discrepancy may result from the difference in co-culture systems. To our knowledge, we incubated for the first time CLL-cells on human BMSCs with different cytokines. The more exact calculation of surviving cell fractions may also have influenced the results; we applied a precise method for the determination of living cells, which included measurement of absolute cell count and staining of the apoptotic cells, as opposed to other studies in which only the viable or apoptotic cell population was stained.[12,13,41] Without the combination of measurement of absolute cell counts and annexin V/PI staining, cells that have been in early apoptosis at the beginning of the culture (annexin V-positive but still of normal cell size) are likely to be neglected because they simply form debris and are therefore undetectable by the end of the co-culture. Furthermore, the rather heterogenic effect of IL-4 on CLL behavior was also concluded from a phase I/II IL-4 therapy study.[42]

In conclusion, our results demonstrate that the poor prognostic CLL can be characterized by its capacity to proliferate in co-culture with stroma and IL-2 and IL-10 and its better resistance to apoptosis in the absence of supporting stroma cells. The inducible proliferation may lead to CLL progression in the bone marrow, as best indicated by lower platelet counts. The involvement of mTOR in CLL-cell proliferation was proved[43,44] and inhibition of mTOR has recently received attention as a potential therapeutic agent in CLL.[45] Our data may help to identify which patients might benefit most from a target specific treatment.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)