B-cell chronic lymphocytic leukemia-derived dendritic cells stimulate allogeneic T-cell response and express chemokines involved in T-cell migration

W. ŁUCZYŃSKI[1*], A. STASIAK-BARMUTA[2], J. PISZCZ[3], E. IŁENDO[4], O. KOWALCZUK[5], M. KRAWCZUK-RYBAK[1]

[1]Department of Pediatric Oncology, e-mail: w.luczynski@wp.pl; [2]Flow Cytometry Unit, [3]Department of Hematology, [4]Department of Pediatric Laboratory Diagnostics, [5]Department of Clinical Molecular Biology, Medical University of Białystok, Poland

Received March 12, 2007

Despite discovery of new therapeutic agents, including nucleoside analogs and monoclonal antibodies, the B-cell chronic lymphocytic leukemia (B-CLL) remains incurable. In recent years, some effort has been made in developing T-cell specific immunity against neoplasmatic cells. Reconstitution of effective costimulation and immunological response of host T-cells against CLL cells could be a potential approach in immunotherapeutic trials. CD40/CD40L system is involved in the survival and proliferation of normal and neoplasmatic B-cells. Some preclinical studies have shown that CD40 stimulation can differentiate leukemic cells into dendritic cells (DCs) and result in host response. In this study, we sought to determine whether B-CLL cells could be turned into efficient and functional antigen presenting cells, as well as to assess the type of allogeneic T-cell response against B-CLL – derived DCs. Material and methods: B-CLL cells from 25 patients were cultured with or without the presence of CD40L and IL-4 for 96 hours and then cultured in mixed lymphocyte reaction with allogeneic T-cells. Results: 1) after CD40 stimulation B-CLL cells achieved phenotypical and functional characterization of DCs (i.e. upregulated co-stimulatory and adhesion molecules at mRNA and protein level) 2) leukemia-derived DCs expressed higher amount of mRNA for chemokines involved in T-cell migration (MDC, TARC and CCR7) 3) the proliferating response of T-cells against leukemia-derived DCs consisted of CD4 and CD8 cells (upregulation of HLA-DR and OX40). Conclusions: our experiment confirm that B-CLL cells can be turned into dendritic-like cells, additionally, these cells express chemokines involved in T-cell migration and stimulate allogeneic response.

Key words: CD40L; chronic lymphocytic leukemia; dendritic cells; T – lymphocytes

Chronic lymphocytic leukemia, a mature B-cell neoplasm is characterized by peripheral blood lymphocytosis and infiltration of the bone marrow by the $CD5^+CD19^+$ cells. It is the most common type of leukemia among adults in the Western world. The profound deregulation of the host immune system in this disease, further exacerbated by therapeutic agents, refers to both cellular and humoral responses and has been intensively studied in the last few years [1]. Despite discovery of some new therapeutic agents, including nucleoside analogs and monoclonal antibodies, the disease remains incurable. Recently, there has been increasing evidence of allogeneic transplantation efficacy, even though this procedure shows also high toxicity and mortality rates. Complete remissions after allogeneic stem cell transplantations suggest susceptibility to the eradication of the B-CLL cells by alloreactive T-cells. In recent years, some effort has been made in developing T-cell specific immunity against neoplasmatic cells in acute and chronic leukemias. In the case of B-CLL and BCP-ALL, the malignant cells are the neoplastic counterpart of a subpopulation of B-cells, so they could function as APCs and present leukemic antigens to the host T-cells. Both CLL and ALL cells express MHC class I and II molecules, although they are not effective as stimulator cells in mixed lymphocyte reactions. The possible mechanisms of this phenomenon may include a weak expression of costimulatory and adhesion molecules on the surface of leukemic cells or the production of

*Corresponding author

Abbreviations: APCs – antigen presenting cells, ALL – acute lymphoblastic leukemia, B-CLL – B-cell chronic lymphocytic leukemia, B-CLL-DCs – B-CLL derived dendritic cells, BCP-ALL – B-cell precursor acute lymphoblastic leukemia, CD40L – CD40 ligand, DCs – dendritic cells, DMSO – dimethylsulfoxide, CTL – cytotoxic T lymphocytes, FBS – fetal bovine serum, IL – interleukin, MHC – major histocompatibility complex, MLR – mixed lymphocyte reaction, TGF – transforming growth factor, TNFR – tumor necrosis factor receptors

Table 1. Clinical characteristics of B-CLL patients

Patients		Age (years, mean ±SD)	WB ($x10^9$/L, mean ±SD)	Hb (g/L, mean ±SD)	PLT ($x10^9$/L, mean ±SD)	β-microglobulin* (g/L, mean ±SD)	LDH** (mkat/l, mean ±SD
M	F						
14 (56%)	1 (44%)	64.5 ±7.7	108.8 ±92.2	115.0 ±24.6	164.2 ±69.1	3977.0 ±1604.9	8.56 ±4.54

* normal range 800-2400
** normal range 0-8

Table 2. Monoclonal antibodies used in flow cytometry

B-CLL CELLS
Costimulatory and adhesion molecules
CD1a, CD11c, CD40, CD42a, CD54, CD58, CD80, CD83 CD86, CD137, CD137L, CD209, HLA I class, HLA-DR
Chemokine receptor
CCR7
T-CELLS (CD4 and CD8)
Activation and costimulatory molecules
CD25, CD28, CD62L, CD69, CD71, CD45RA, CD45RO, CD95, CD134, CD137, CD152, CD154, HLA-DR
Chemokine receptor
CCR7

immunosuppressive cytokines such as TGF-β and IL-10 [2]. Reconstitution of effective costimulation and immunological response of host T-cells against CLL cells could be a potential approach in immunotherapeutic trials. CD40, a member of TNFR family, is deeply involved in the survival and proliferation of normal and neoplasmatic B-cells. Some preclinical studies have shown that CD40 stimulation can differentiate ALL and CLL cells into dendritic cells and result in host response [3–7]. The optimum reaction of T-cells depends not only on increased immunogenicity of leukemic cells but also on their ability to recruit effector cells to the tumor sites. This could be caused by the production of specific chemokines in leukemia-derived dendritic cells [8]. However, whether leukemia-derived DCs acquire the full functional phenotype and capacities of APCs is still unknown.

In this study, we sought to determine whether B-CLL cells could be turned into efficient and functional APCs. We also tried to assess the type of allogeneic T-cell response against B-CLL derived DCs.

Material

The study was approved by the Review Board**,** Medical University of Białystok, Poland. After informed consent, peripheral blood samples were obtained from 25 untreated patients with B-CLL and from 1 healthy donor. The diagnosis of B-CLL was based on standard clinical and laboratory criteria. Characteristics of the patients studied are summarized in Table 1.

Methods

Cells and culture. Peripheral blood mononuclear cells (PBMC) of B-CLL patients were isolated by Ficoll density separation, cryopreserved in Freezing Container (Sigma) with RPMI-1640/FBS/DMSO (50/40/10%) and stored in liquid nitrogen. After thawing, B-CLL cells were cultured with a medium (containing RMPI-1640-90% and FBS-10%) at 37^0C in a 5%CO_2 humidified atmosphere in 24-well plates (Beckton-Dickinson, USA) for 96 hours at a concentration of 10^5 cells/well in a total volume of 1ml/well. The cells were cultured with or without the presence of the combination of CD40L (3μg/ml, a gift from Amgen, USA) and IL-4 (80ng/ml, Sigma-Aldrich, USA). After 96 hours of culture, B-CLL cells were harvested and washed for further experiments and analysis. As assessed by flow cytometry, more than 90% of the cells from B-CLL patients before and after the culture were CD19 and CD5 positive. Dendritic cells generated from B-CLL patients are further called B-CLL-DCs.

Mixed lymphocyte reaction (MLR). T-cells were purified from normal donor by negative selection using Pan T-cell isolation kit (Miltenyi), according to the producer's instruction. Purity of cell fractions in all experiments was greater than 95% as assessed by flow cytometry analysis ($CD3^+$). T-cells (effectors, 1 x 10^5 cells/well) were co-cultured for 96 hours with:

1) "medium" alone
2) γ-irradiated (30 Gy) B-CLL cells previously treated with medium and CD40L/IL-4 (B-CLL-DCs)
3) γ-irradiated (30 Gy) B-CLL cells previously treated with medium alone
4) concavalin A as a positive control (5mg/ml)

in a final volume of 0.2mL per well. B-CLL-DCs were added to the T-cells at DC:T cell ratio 1:3. The co-cultures were pulsed with [^{3}H] thymidine (1mCi/well, Amersham) for the final 18 hours of culture. Thymidine incorporation was determined on day 5 by liquid scintillation β-emission and reported as mean cpm from triplicate cultures.

Flow cytometry analysis. Expression of cell surface molecules was determined by flow cytometry using standard methodology. All monoclonal antibodies used in our study are listed in Table 2 and were purchased from Beckman Coulter and Beckton Dickinson (only CCR7). In all experiments, isotype controls were included using appropriate PE-, FITC- or PerCP-conjugated irrelevant monoclonal antibodies of the same Ig class or subclass. A total of 10^5 cells were incubated

Table 3. Names, symbols and IDs of APM genes assessed in the study – according to the TaqMan Gene Expression Assays (Applied Biosystems).

Gene symbol, alias	Gene name	Assay ID
B-CLL CELLS		
	Costimulatory and adhesion molecules	
CD40	CD40 antigen (TNF receptor superfamily member 5)	Hs00374176_m1
CD54 / ICAM-1	intercellular adhesion molecule 1 (CD54)	Hs00164932_m1
CD80	CD80 antigen (CD28 antigen ligand 1, B7-1 antigen)	Hs00175478_m1
CD83	CD83 antigen (activated B lymphocytes, immunoglobulin superfamily)	Hs00188486_m1
CD86	CD86 antigen (CD28 antigen ligand 2, B7-2 antigen)	Hs00199349_m1
	Chemokines	
CCR7	chemokine (C-C motif) receptor 7	Hs00171054_m1
TARC / CCL17	chemokine (C-C motif) ligand 17	Hs00171074_m1
MDC / CCL22	chemokine (C-C motif) ligand 22	Hs00171080_m1

Table 4. mRNA expression for costimulatory/adhesion molecules and chemokines assessed in B-CLL cells before and after the culture with or without CD40L/IL-4.

	I	**II**	**III**	**Statistics**		
	after thawing	**cultured with medium + CD40L + IL-4**	**cultured with medium only**	I vs II	I vs III	II vs III
	$2^{-\Delta\Delta CT}$ mean	$2^{-\Delta\Delta CT}$ mean	$2^{-\Delta\Delta CT}$ mean			
CD40	1.47	4.92	2.40	(-)	(-)	(-)
CD54	8.82	15.80	2.11	**p<0.01**	(-)	**p<0.01**
CD80	0.40	45.95	1.59	**p<0.05**	(-)	**(-)**
CD83	79.21	984.16	8.61	(-)	(-)	**p<0.01**
CD86	27.34	373.93	5.30	(-)	(-)	(-)
CCR7	148.58	10733.50	89.50	**p<0.01**	(-)	(-)
MDC	4.64	55.26	0.10	**p<0.0001**	(-)	**p<0.0001**
TARC	319.45	1213.83	17.21	**p<0.0001**	(-)	**p<0.0001**

with monoclonal antibodies for 30 min at 4^0C, washed twice, and analyzed on a Beckman Cytomics FC 500 MPL. Percentages of positive cells were calculated. Results are expressed as percentage of positive cells (%) among stained cells.

Real-time quantitative RT-PCR. The mRNA was isolated from mononuclear cells before and after the culture using Dynabeads mRNA Direct Micro Kit (Dynal) in accordance with the manufacturer's instructions. The first strand of cDNA was synthesized using random hexamers as primer and High Capacity cDNA Archive Kit by Applied Biosystems. Real-time PCR analysis was performed using TaqMan Low Density Arrays (Applied Biosystems). Real-time PCR primers and probes ("TaqMan Gene Expression Assays") for the assessed genes listed in Table 3 were spotted onto 96-well card (Applied Biosystems). To normalize the amount of expressed mRNAs, the internal housekeeping gene GAPDH was used. Each sample was measured in quadruplicate in a single RT-PCR run. Thermal cycling and fluorescence detection were performed on Applied Biosystems ABI Prism 7900 HT Sequence Detection System with ABI Prism 7900 HT SDS Software 2.1. To calculate our data we used Comparative C_t method for relative quantification ($\Delta\Delta C_t$ method) which describes the change in expression of the target gene in a test sample relative to a calibrator sample and provides accurate comparison between the initial level of template in each sample. As a calibrator sample we used Total Raji RNA by Applied Biosystems which was processed in the same way as the test samples.

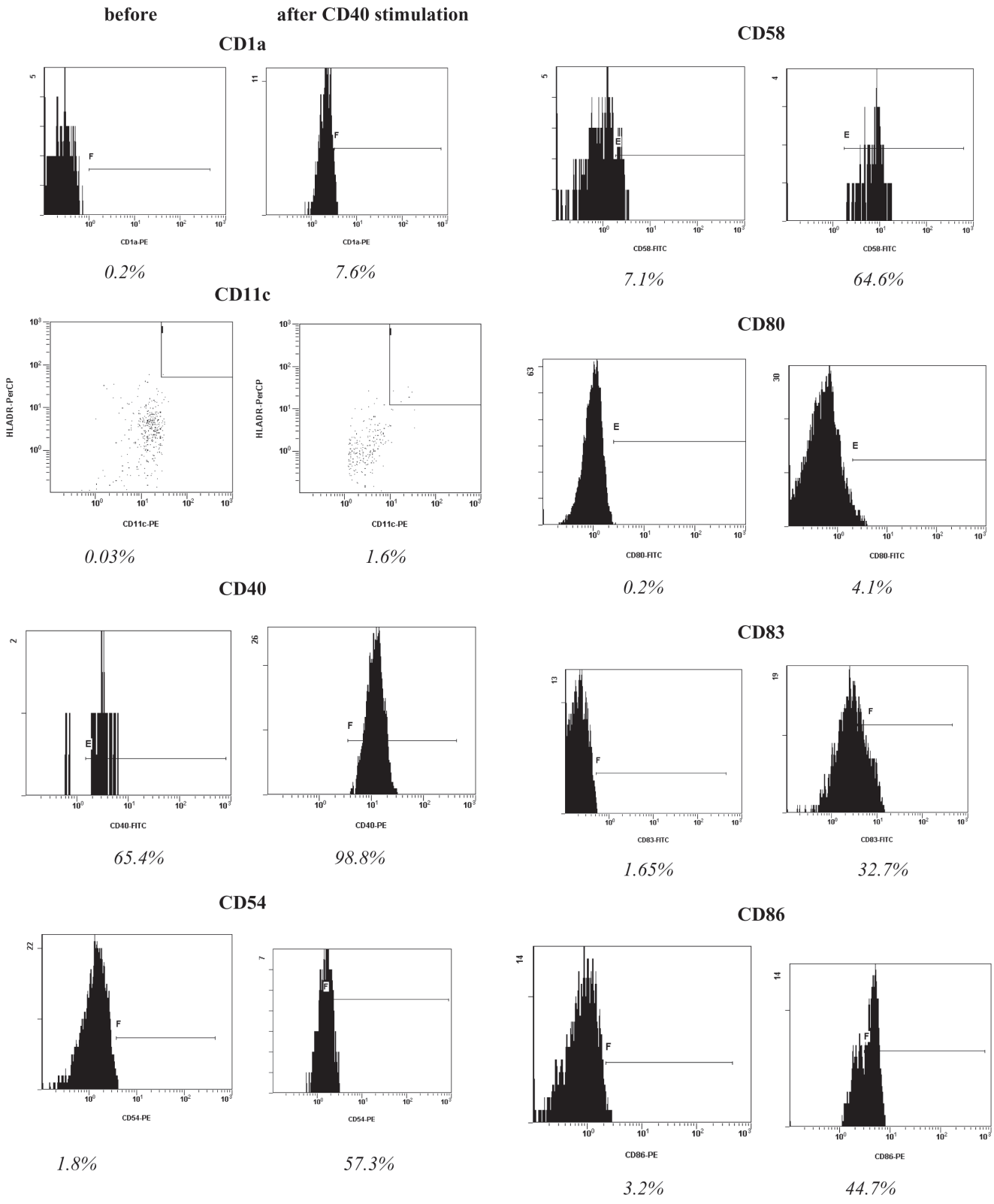
Statistics

Statistical analysis was performed using Statistica 6.0 for Windows. The results were not normally distributed and are expressed as mean, median, standard error (SE), standard deviation (SD) and 25^{th}-75^{th} percentiles. Significance levels were calculated according to the nonparametric Wilcoxon test (dependent subgroups) and the Mann-Whitney *U* test (independent subgropups). The level of $p<0.05$ was regarded as significant.

Results

B-CLL cells can be differentiated into dendritic-like cells. Main results concerning expression of costimulatory and adhesion molecules obtained from real-time RT-PCR are shown in Table 4 and from flow cytometry – in Table 5.

1) Real-time RT-PCR – mRNA level


before
after CD40 stimulation
CD1a
CD1a-PE
0.2%
7.6%
CD58
CD58-FITC
7.1%
64.6%
CD11c
HLADR-PerCP
CD11c-PE
0.03%
1.6%
CD80
CD80-FITC
0.2%
4.1%
CD40
CD40-FITC
CD40-PE
65.4%
98.8%
CD83
CD83-FITC
1.65%
32.7%
CD54
CD54-PE
1.8%
57.3%
CD86
CD86-PE
3.2%
44.7%

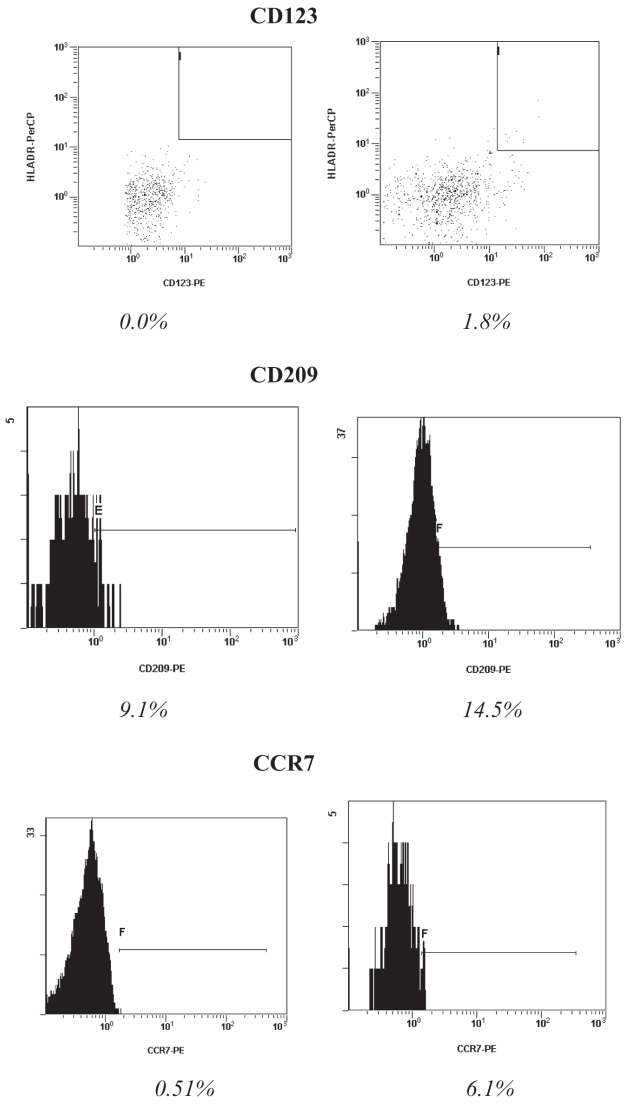


Figure 1. Percentages of B-CLL cells before and after CD40L/IL-4 stimulation – an example

Before the culture mRNA expression for costimulatory and adhesion molecules in B-CLL cells was low.

After CD40 stimulation we noted a rise in the expression of mRNA for all assessed molecules but only in the case of CD54 and CD80 it was statistically significant. Additionally, the expression of mRNA for CD54 and for CD83 was significantly higher after the culture with CD40L and IL-4 than after the culture with medium alone.

2) Flow cytometry – protein level

After thawing, before the culture, B-CLL cells were mostly negative for CD1a, CD11c, CD42a, CD54, CD80, CD83, CD123, CD137, CCR7 (<1.0% positive cells), moderately positive for CD58, CD86 and CD209 (5-25%) and highly positive for CD40, CD137L, HLA class I and II (>60%).

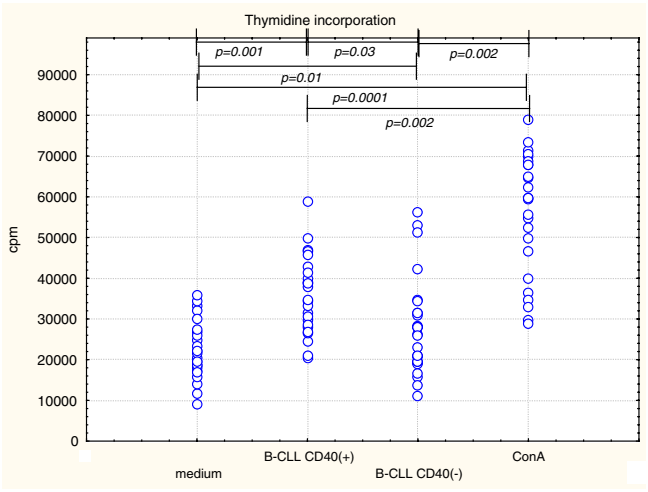


Figure 2. Thymidine incorporation in T-cells after culture with medium. CLL cells after CD40L/IL-4 stimulation (B-CLL CD40+), CLL cells (B-CLL CD40-) and Concavalin A (ConA).

After the culture, a statistically significant upregulation of virtually all important costimulatory and adhesion molecules was noted – example – Fig 1. In some populations this increase was observed also in cells treated with medium alone, i.e. CD1a, CD54, CD80, CD83, HLA class I. However, in most of the cases we found also statistically significant differences between the percentages of positive cells treated with CD40L/IL-4 vs. those treated with medium alone (i.e. CD11c, C42a, CD54, CD58, CD80, CD86, CD123, CD209 and HLA I class).

B-CLL – DCs express chemokines involved in T-cell migration. We noted higher mRNA levels of chemokines CCR7, MDC and TARC in B-CLL cells after CD40 stimulation as compared to those obtained before the culture and after the culture with medium alone. These differences were statistically significant (see Table 4). The expression of CCR7 on the cell surface of B-CLL cells, assessed by flow cytometry, rose also after CD40 stimulation (Table 5).

B-CLL – DCs stimulate allogeneic T-cell response

1) [^{3}H] thymidine incorporation

Results from this part of the experiment are shown in Fig. 2. The proliferation of T-cells was the highest after stimulation with concavalin A (positive control), it was higher also after MLR with B-CLL cells treated with CD40L and IL-4 than treated with medium alone ($p<0.05$).

2) Flow cytometry

Only statistically significant differences between group 2 (T-cells cultured with B-CLL-DCs) and 3 (T-cells cultured with B-CLL cells) are mentioned in this section. The expression of CD134 (OX40) and HLA-DR on T-cells (both CD4 and CD8) was higher after MLR with B-CLL-DCs (group 2) than after MLR with B-CLL cells treated with medium alone (group 3, see Fig. 3). Percentages of T-cells with CD134 and

Table 5. Percentages of B-CLL cells with costimulatory/adhesion molecules and chemokine expression before and after the culture with or without CD40L/IL-4 assessed with flow cytometry.

	I	II	III	Statistics		
	after thawing	cultured with medium + CD40L + IL-4	cultured with medium only	I vs II	I vs III	II vs III
	median (25^{th}-75^{th} percentile)	median (25^{th}-75^{th} percentile)	median (25^{th}-75^{th} percentile)			
CD1a	0.1 (0.1÷1.3)	5.0 (0.1÷11.0)	3.5 (0.1÷36.0)	**p=0.0059**	**p=0.009**	(-)
CD11c	0.1 (0.1÷0.3)	2.3 (1.1÷3.05)	0.1 (0.1÷0.35)	**p=0.0001**	(-)	**p=0.0001**
CD40	98.0 (95.0÷99.0)	62.0 (51.5÷80.0)	73.0 (58.0÷84.5)	**p=0.0005**	**p=0.0005**	(-)
CD42a	0.1 (0.1÷0.1)	0.1 (0.1÷4.0)	0.1 (0.1÷0.2)	**p=0.0069**	(-)	**p=0.009**
CD54	1.7 (0.4÷8.0)	48.5 (31.0÷68.5)	28.0 (14.5÷58.0)	**p=0.0001**	**p=0.0008**	**p=0.007**
CD58	14.0 (6.0÷28.0)	56.0 (36.0÷78.0)	32.0 (16.0÷67.0)	**p=0.0001**	(-)	**p=0.01**
CD80	0.1 (0.1÷0.1)	5.0 (2.0÷9.5)	0.1 (0.1-2.5)	**p=0.0001**	**p=0.002**	**p=0.0002**
CD83	1.7 (0.85÷6.7)	33.5 (24.0÷44.5)	30.5 (20.5÷45.0)	**p=0.0001**	**p=0.0001**	(-)
CD86	8.0 (2.4÷13.0)	28.0 (24.5÷43.0)	7.0 (5.9÷15.5)	**p=0.0001**	(-)	**p=0.0001**
$CD80^+CD86^+$	0.1 (0.1÷0.1)	5.0 (3.0÷10.0)	0.25 (0.1÷4.0)	**p=0.0001**	**p=0.002**	**p=0.003**
CD123	0.1 (0.1÷0.1)	2.25 (1.1÷3.7)	0.1 (0.1÷0.1)	**p=0.0001**	(-)	**p=0.0001**
CD137	0.3 (0.1÷76.5)	14.0 (5.0÷20.0)	11.0 (0.1÷32.0)	(-)	(-)	(-)
CD137L	75.0 (61.0÷94.0)	68.0 (42.0÷82.0)	56.0 (21.0÷76.0)	(-)	(-)	(-)
CD209	4.0 (1.3÷10.5)	20.0 (12.0÷30.0)	8.0 (0.1÷16.0)	**p=0.002**	(-)	**p=0.001**
HLA class I	98.0 (95.0÷100.0)	97.0 (83.5÷100.0)	87.0 (75.5÷95.0)	(-)	**p=0.003**	**p=0.01**
HLA-DR	47.0 (36.0÷81.0)	70.0 (36.0÷78.0)	66.0 (31.0÷76.0)	(-)	(-)	(-)
CCR7	0.5 (0.1÷1.8)	4.3 (0.1÷8.0)	0.1 (0.1÷4.0)	**p=0.02**	(-)	**p=0.003**

HLA-DR expression were low before the culture and very high after the culture with concavalin A (control stimulator). For instance: $CD4^+$ cells expressed CD134 and HLA-DR: before MLR 3.65% and 5.43% (mean, respectively), after MLR with B-CLL-DCs 33.71% and 19.85%, after MLR with B-CLL cells – 16.50% and 10.57%, and after ConA – 32.17% and 23.76%.

Discussion

To test the hypothesis that leukemic cells can be turned into dendritic-like cells we stimulated B-CLL cells with CD40L/IL-4 and cultured them with allogeneic T-cells in mixed lymphocyte reaction. The upregulation of costimulatory/adhesion molecules and markers of DCs (CD1a, CD11c, CD54, CD80, CD83, CD86, CD123) noted in our experiment at mRNA and protein level in leukemic cells after CD40 ligation has been observed by several authors [3, 9, 10]. The results obtained from real-time RT-PCR and flow cytometry have confirmed that leukemic cells can achieve the phenotype of dendritic-like cells. The combination of stimulators i.e. CD40L and IL-4 used in our study was chosen after an intensive review of the literature and is regarded as the strongest in upregulation of costimulatory and adhesion molecules on B-CLL cells [7, 9, 10]. However, transduction of CLL cells with CD40L by viral vectors represents an alternative strategy to enhance their immunogenicity. In one study, CD40L-transduced CLL cells were superior in inducing T-cell response in comparison to CD40L-stimulated CLL cells [11]. In another – B-CLL cells were genetically manipulated to express TRICOM costimulatory molecules: B7-1, ICAM-1 and LFA-3 [12]. TRICOM-infected CLL cells stimulated the autologous T-cell proliferative and cytotoxic activity. In the authors' opinion, all molecules contributed to the enhanced immunogenicity of B-CLL cells. It is difficult to estimate which costimulatory molecules are the most important in the function of B-CLL-DCs. Leukemia-derived dendritic cells with the coexpression of CD80 and CD86 are classified as mature DCs and induce robust allogeneic T-cell proliferation [13]. In our study the $CD80^+CD86^+$ population rose significantly after CD40 stimulation. Another marker of DC maturation is the DC-SIGN (CD209), which plays an important role in DC-induced adhesion, migration and activation of T-cells, and participates in the immune escape in cancer. According to Zhou et al., one of the possible mechanisms of escape from immune surveillance by tumor cells can be the suppression of DC maturation by DC-SIGN blockade [14]. We noted a rise in the expression of CD209 on CLL cells after C40 stimulation – this can also have a positive effect in future immunotherapy, e.g. in renal cell carcinoma, where $CD209^+$ DCs infiltrate tumor after immunotherapy with IL-2, IFN-α and GM-CSF, thus suggesting clinical potential of this therapeutic approach [15].

A major function of "normal" DCs is to migrate to lymph nodes to get in contact with T-cells. This process is regulated by CCL21/CCL19/CCR7 axis (CCR7 is usually present on mature dendritic cells). In our study, B-CLL cells showed upregulation of all elements of this axis after CD40 stimulation. Also CD40L-activated BCP-ALL cells expressed CCR7 and showed migration in response to CCL19. These cells

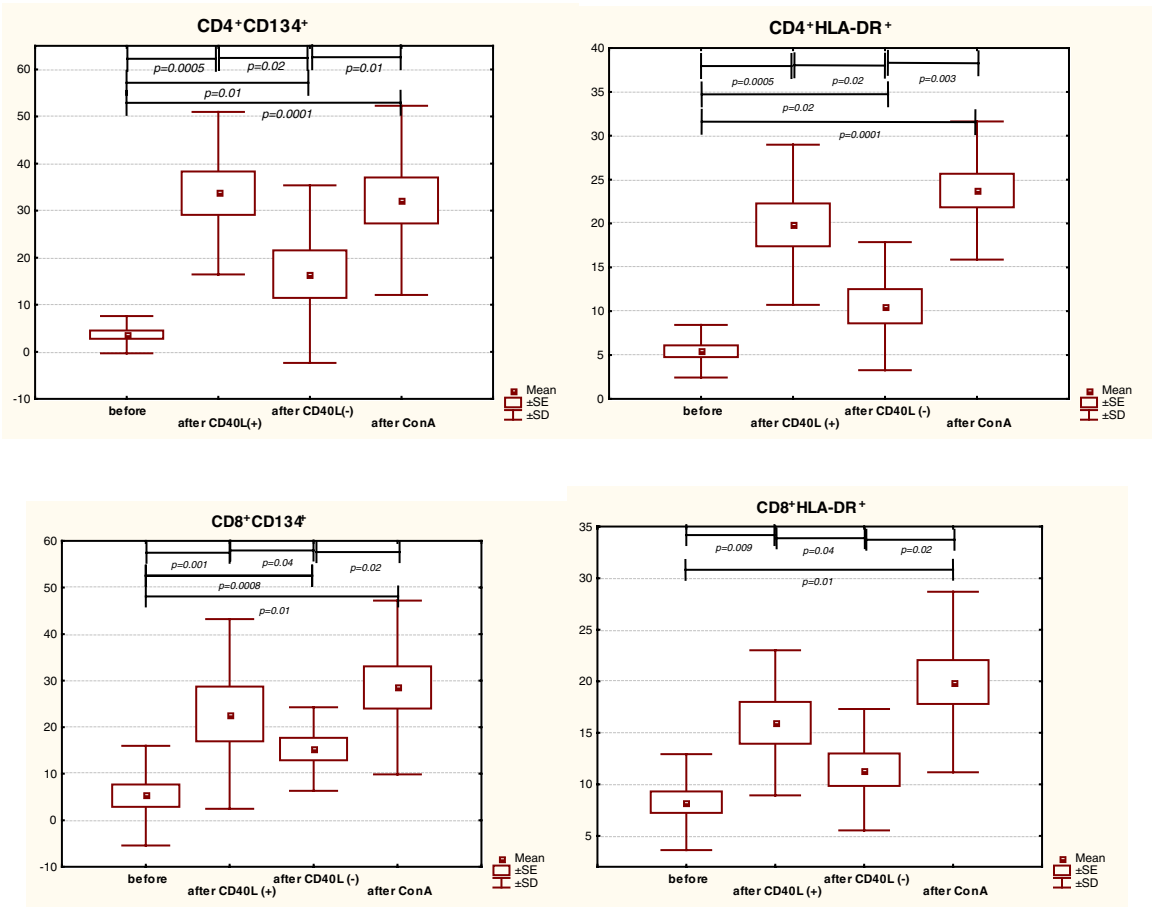


Figure 3. Percentages of T-cells with the coexpression of CD134 and HLA-DR after MLR.

stimulated T-cell response, but did not sustain proliferation over time and T-cells became anergic [3]. In the authors' opinion, this was caused by the lack of IL-12 production in BCP-ALL-DCs and addition of exogenous IL-12 reverted anergy of T-cells. In a few other studies, CD40-stimulated CLL and ALL cells produced CC-chemokines: MDC and TARC, and supported the transendothelial migration of antileukemia T-cells [8, 16]. Higher production of chemokine receptor CCR7 and CD62L (L-selectin) on leukemia-derived dendritic cells was also demonstrated by Mohty et al. in other lymphoid malignancies i.e. ALL, CLL and plasma cell leukemia [9]. In another study, CD40 stimulation induced higher production of CCR7 in myeloid leukemia-derived dendritic cells [17]. In animal model, MDC gene transfer resulted in antitumor activity of $CD8^+$ cells [18]. We suppose that higher expression of chemokines involved in T-cell migration by leukemia-derived DCs can be an additional, positive effect of CD40 stimulation. However, some authors are reporting higher production of TARC by Reed-Sternberg cells in Hodgkin's lymphoma. In B-cell lymphoma, MDC (CCL22) secretion was involved in the chemotaxis and migration of intratumoral T(reg) cells ($CD4^+CD25^+$) which have a suppressory influence on other T-cells [19, 20].

Leukemia-derived dendritic cells, if they stimulate T-cell response and result in elimination of the neoplasmatic clone, could be used in future immunotherapy. In order to assess this response we examined the nature of allogeneic T-cell repertoire induced by B-CLL-DCs and "normal" B-CLL cells. In our study, leukemia-derived DCs induced a proliferative response of allogeneic T-cells separated from healthy donors. If these T-cells are to be useful for adaptive immunotherapy in B-CLL, they should exhibit an activated phenotype. It is interesting which cells act as antileukemic clones: $CD4^+$ or $CD8^+$. In our experiment, both $CD4^+$ and $CD8^+$ cells showed activation with B-CLL-DCs after MLR, but most authors consider $CD8^+$ cells as CLL-reactive lymphocytes [10]. These CTL lines effectively kill leukemic cells (also not changed with CD40L/IL-4 – "normal" B-CLL cells). According to Buhmann et al., the character of the immune response against the neoplastic cell clone, depends on the MHC background against which the CLL cells are presented: the CD40L-activated CLL cells induced the expansion of allogeneic $CD8^+$

cells (CTL) and in contrast, the autologous – the expansion of $CD4^+$ Th_1 lymphocytes [21].

We showed upregulation of HLA-DR and OX40(CD134) on $CD4^+$ and $CD8^+$ T-cells with B-CLL-DCs after MLR. The signal resulting from the ligation of OX40L (expressed on APCs) with OX40 (on T-cells) facilitates the clonal expansion and generation of $CD4^+$ memory antigen-specific T-cells, even though OX40 functions as a costimulatory molecule not only for $CD4^+$ cells but also for $CD8^+$ T-cells [22, 23]. The molecular transfer of the combination of CD40L and OX40L to B-CLL cells expanded the autologous response in both $CD4^+$ and $CD8^+$ subpopulations, but only $CD4^+$ (and not $CD8^+$) cells showed upregulation of OX40 after the culture with modified B-CLL cells [23]. Possibly, the activation of OX40 could lead to the restoration of antitumor immunity. One of the most important complications of allogeneic transplantation, i.e. acute graft-versus-host disease, probably accompanying a graft-versus-leukemia effect, is also mediated by the activated donor T-cells. Some authors have shown that this activation is presented by upregulation of CD25, CD69 and CD134 both on $CD4^+$ and $CD8^+$ cells [24]. Also other reports suggest that $CD134^+$ T-cells are involved in chronic graft-versus-host disease [25].

One of the major issues in cancer immunotherapy is to maintain the immune response against leukemic cells. After vaccination with B-CLL cells expressing CD40L and IL-2, T-cell reactivity was only transient, probably due to a rise in the immunoregulatory cells ($CD4^+CD25^+LAG\text{-}3^+FoxP\text{-}3^+$) [26]. The authors propose removal of these cells from future immunotherapeutic trials. In another experiment, the systemic response to CD40L/IL-2 expressing leukemic cells involved an increase in circulating $CD3^+$/$HLA\text{-}DR^+$ lymphocytes. Vaccination resulted in a rise in leukemia-reactive both $CD4^+$ and $CD8^+$ cells**.** The production of antibodies was also observed, and like in our experiment, no significant increase was found in the $CD4^+CD25^+$ population [5].

In antileukemic immunity, not only T-cells but also NK lymphocytes play a role [27]. T-cell independent approach in immunotherapy can be important in patients with a decreased number of T-cells after chemotherapy. The use of autologous DCs pulsed with leukemic tumor lysate can be another method of immunotherapy in CLL [28]. The stimulatory effect of the cells prepared in this manner was even enhanced with priming CLL cells with CD40L and IL-4. The "preactivation" of autologous T-cells with the use of phorbol 12-myristate 13-acetate (PMA) and ionomycin is a totally different approach [29]. This simple assay results in enhanced antigen-presenting capacity and susceptibility to apoptosis of CLL cells. Recently, also Toll-like receptor agonists (e.g. imidazoquinolines and guanosine analogs) have shown activity in enhancing the immunogenicity of CLL cells [30].

The preliminary results of our investigations as well as results of other authors are promising. However, there are several questions still to be answered, e.g. the therapeutic value of these approaches [7]. Clinical experience in CD40L-based immunotherapy in leukemias is scarce, with both discouraging and encouraging results [5, 6]. To conclude, the data presented in this study support the hypothesis that T-cell targeting immunotherapy could be a promising therapeutic option in CLL. It is clear that CD40-based strategies will develop in immunotherapeutic trials in acute and chronic leukemias both in children and adults.

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