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Acute lymphoblastic leukemia-derived dendritic cells express tumor associated antigens: PNPT1, PMPCB, RHAMM, BSG and ERCC1.

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In all types of leukemia both in children and adults there is a need for novel therapies that could reduce the risk of relapse after standard treatment. Acute lymphoblastic leukemia (ALL) cells are ineffective antigen presenting cells, but as shown by many authors including results from our laboratory, stimulation with CD40L restores their antigen expressing capacity. The development of T-cell therapies for leukemic patients can be based on discovery of leukemia-associated antigens (LAA) which could be recognized by the host immune system. The aim of our present study was to test the hypothesis that leukemia-derived dendritic cells maintain the expression of tumor associated antigens. Twenty five children with B-cell precursor ALL were prospectively enrolled into the study. The mononuclear cells from peripheral blood or bone marrow were cultured and stimulated (or not) with CD40L and IL-4. The assessment of costimulatory/adhesion molecules with the use of flow cytometry and real-time RT PCR were used to confirm the possibility of turning ALL cells into dendritic-like cells. Additionally 22 tumor associated antigens mRNA levels were determined by real-time PCR technique with the TaqMan chemistry using ready-to-use Low Density Arrays for Gene Expression. The results of the study showed maintained expression and even up-regulation of some (PNPT1, PMPCB, HMMR/RHAMM, BSG and ERCC1) tumor associated antigens in CD40-activated leukemic cells. CD40L stimulation leading to the differentiation of leukemic cells into DCs which combine both antigen presenting function and expression of tumor associated antigens represents an interesing approach in cancer immunotherapy.

Key word: acute lymphoblastic leukemia, children, dendritic cells, tumor associated antigens, immunotherapy

Acute lymphoblastic leukemia (ALL) constitutes about 30% of all neoplasms in children and only about 1% in adults. Two thirds of all cases occur below the 18th year of age. The current treatment of the disease include chemo-, radiotherapy and in some cases - allogeneic bone marrow transplantation. In spite of high rate of cure in children (~80%) and a moderate in adults (~40%), a significant number of patients develop refraction to the chemo- and radiotherapy. An allogeneic transplantation, offering the possibility for break-through the chemoresistance, can be related to acute and chronic complications and disturbances in a quality of life. Thus in all types of leukemia both in children and adults, there is a need for novel therapies that could reduce the risk of relapse after standard treatment. One of the promising options is the use of immunotherapy to stimulate the patient's immune system to recognize and destroy leukemic cells. Dendritic cells (DC), extensively examined in this field could be used as tumor antigens presenting cells leading to the initiation of anti-leukemic response. For this purpose DCs can be loaded with leukemia antigens or leukemic blasts can be turned into DCs [1]. ALL cells are ineffective APCs, but as shown by many authors including results from our laboratory, stimulation with CD40L restores their antigen expressing capacity [2, 3]. The CD40 ligation represents the most valuable, sustained model system for ALL cell conversion into DC [4–6]. However, whether leukemia-derived DC acquire the full functional phenotype of APC is still unknown.

From the other point of view the development of T-cell therapies for leukemic patients can be based on discovery of leukemia-associated antigens (LAA) which could be

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Abbreviations: ALL – acute lymphoblastic leukemia, ALL-DCs – acute lymphoblastic leukemia-derived dendritic cells, AML – acute myeloid leukemia, APC – antigen presenting cell, CLL – chronic lymphocytic leukemia, DCs – dendritic cells, LAA – leukemia associated antigen, MRD – minimal residual disease, TAA – tumor associated antigen

recognized by the host immune system. TAAs based immunotherapies can represent a novel treatment for leukemias including most common: ALL, AML and CLL. The idea of tumor-associated antigens (TAA) assumes that some antigens are expressed in cancer cells but not (or only at low level) in normal tissues [7]. Ideal targets for TAA would include antigens exclusively expressed in tumor cells in both primary and metastatic disease. Progress in identifying such markers for leukemias has been improved in the last few years [8–10]. The identification of leukemia-associated antigens (LAA), essential for the future development of effective immunotherapeutic strategies is difficult because leukemic cells originate from normal lymphocyte presursors. Even though many tumor associated antigens are known only few of them are considered as leukemia-associated antigens [11]. TAAs can represent different elements of cell structure and function but many of them are involved in DNA proliferation/repair system like ERCC1 and PCNA [12]. In myeloid leukemias LAAs include: heat shock proteins HSJ2 and HSP70, the M-phase phosphoprotein 11 (MPP11), the BRCA-1 associated protein and RHAMM [11]. RHAMM, fibromodulin, PRAME and MPP11 are expressed in CLL patients, but not in healthy volunteers [8]. Lately some clinical trials including the idea of using TAA in cancer immunotherapy are ongoing. To date, the assessment of TAA in leukemia derived dendritic cells was performed in AML and CLL leukemias [13, 14]. Little is known about the changes in LAAs gene expression in acute lymphoblastic leukemia. The aim of our present study was to test the hypothesis that ALL-DCs maintain the expression of tumor associated antigens.

Materials and methods

Patients. Children with B-cell precursor acute lymphoblastic leukemia (BCP-ALL) were prospectively enrolled into the study (n = 25, 14 boys, 11 girls, aged 6.35 ± 3.8).Leukemia was diagnosed when neoplastic infiltration of bone marrow was greater than 30%. Patients were treated according to the protocol recommended by the Polish Pediatric Leukaemia/Lymphoma Study Group – ALL IC 2002. Peripheral blood samples or bone marrow (10ml) were taken at the time of diagnosis, before any treatment. The institutional review board approved this study and informed consent was obtained from patients and their parents.

Cells and culture conditions. Mononuclear cells from bone marrow or peripheral blood samples were isolated after centrifugation over a Ficoll density gradient (Histopaque 1077, Sigma), washed three times in phosphate-buffered saline (PBS) and immediately cryopreserved in RPMI-1640 50%, fetal bovine serum (FBS) 40% and dimethyl sulfoxide (DMSO) 10% (all purchased from Sigma). Vials were first placed in a Freezing Container (Sigma) at –80°C for 24 h and then stored in liquid nitrogen. After thawing ALL cells were washed, resuspended in medium contaning RPMI-1640 90%/FBS 10% („medium"), cultured in 24-well flat-bottom plates at a concentration of 1×10^5/ml and stimulated (or not) with CD40L (3 μg/ml, a kind gift of Immunex/Amgen, USA) and IL-4 (80 ng/ml, Sigma) (CD40L+IL-4 = „CD40 ligation"). This combination was chosen according to our previous data and is the most often used in such experiments. The cells were incubated in a total volume of 1 ml/well at 37°C in 5% CO_2 for 96 h. On day 5 (after culture) the cells were harvested and concentrated for flow cytometry and mRNA studies. The trypan blue dye (Sigma) exclusion assay was used to study viability, all assessed samples had at least 80% viable cells before and after culture.

Flow cytometry. Immunophenotyping of the cells was performed as previously described [2]. In brief, fluorescein- (FITC) or phycoerythrin- (PE) or peridinin chlorophyll protein (PerCP)-conjugated mouse monoclonal antibodies (and isotype controls) to the following antigens were used: CD1a, CD10, CD11c, CD19, CD34, CD40, CD45, CD54, CD79a, CD80, CD83, CD86, CD123, HLA class I and II (Becton Dickinson). CD1a and CD83 were considered as DC markers. CD40, CD54, CD80, and CD86 are costimulatory and adhesion molecules. The expression of CD10, CD19, CD34, CD45, and CD79a antigens was used to document the leukemic origin of the dendritic-like cells obtained after the culture with CD40L/IL-4. The mean percentage of positive cells and the mean channel fluorescence of signal (data not shown) were calculated.

Real-time RT-PCR. The mRNA was isolated from mononuclear cells using Dynabeads mRNA Direct Micro Kit (Dynal) according to the producer instruction. First strand cDNA was synthesized using random hexamers as primer and High Capacity cDNA Archive Kit by Applied Biosystems. Twenty two tumor associated antigens (listed in Table 1) and five costimulatory molecules (CD1a, CD54, CD80, CD83, CD86) mRNAs levels were determined by real-time PCR technique with the TaqMan chemistry using ready-to-use Low Density Arrays for Gene Expression by Applied Biosystems. It contained target-specific primers and probe and TaqMan Universal Master Mix, containing AmpErase uracil-N-glycosylase (UNG) to prevent the re-amplification of carryover PCR products. The PCR amplification and fluorescence data collection were performed with ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). To normalize the amount of expressed cytokine mRNAs, the internal housekeping gene GAPDH was used and each complementary DNA (cDNA) product was tested in quadruplicates for each of the twenty two TAAs and five costimulatory/adhesion molecules mRNA and GAPDH mRNA. 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 [15]. As a calibrator sample we used Total Raji RNA by Applied Biosystems which was processed in the same way as the test samples. Data were analyzed with Sequence Detector System (SDS) software version 2.1 (Applied Biosystems). The example of amplification plot for GAPDH is shown in Fig. 1.

Table 1 Names, symbols and IDs of TAA genes assessed in the study according to the TaqMan gene expression assays (Applied Biosystems).

Gene symbol, alias	Gene name	Assay ID	Presence in ALL specimens
PRAME	preferentially expressed antigen in melanoma	Hs00196132_m1	+
PNPT1	polyribonucleotide nucleotidyltransferase 1	Hs00396742_g1	+
HSPA4L	heat shock 70kDa protein 4-like	Hs00204666_m1	-
PMPCB	peptidase (mitochondrial processing) beta	Hs00188704_m1	+
WT1	Wilms tumor 1	Hs00240913_m1	+
HMMR	hyaluronan-mediated motility receptor RHAMM	Hs00234864_m1	+
STEAP1	six transmembrane epithelial antigen of the prostate 1	Hs00185180_m1	-
STEAP3	STEAP family member 3	Hs00217292_m1	+
STEAP 4	STEAP family member 4	Hs00226415_m1	-
BSG	basigin	Hs00174305_m1	+
FMOD	fibromodulin	Hs00157619_m1	+
BIRC5	survivin	Hs00153353_m1	+
PCNA	proliferating cell nuclear antigen	Hs00427214_g1	+
ERCC1	excision repair cross-complementing rodent repair deficiency	Hs00157415_m1	+
JUNB	jun B proto-oncogene	Hs00357891_s1	+
RAF1	v-raf-1 murine leukemia viral oncogene	Hs00234119_m1	+
SSX1	synovial sarcoma, X breakpoint 1	Hs00846692_s1	-
SSX2	synovial sarcoma, X breakpoint 2	Hs00817683_m1	-
BAGE	B melanoma antigen	Hs02380703_g1	+
LIMS1/PINCH	LIM and senescent cell antigen-like domains 1	Hs00192181_m1	-
MAZ	MYC-associated zinc finger protein	Hs00159087_m1	+
MAGE-A3	melanoma antigen family A, 3	Hs00366532_m1	-

Statistical analysis. The results were entered into the database in Microsoft Access 2007 and after the completion transferred and analysed in Statistica 8.0 for Windows (StatSoft, Poland) and GraphPad Prism 5.0 (GraphPad Software, USA). Due to asymmetric data distribution (Kolmogorov-Smirnov and Shapiro-Wilk tests) non-parametric tests were used. Results are expressed by median. Significance levels were calculated according to the nonparametric Wilcoxon test (differences between mRNA levels and percentages of the cells in flow cytometry before and after the culture with or without CD40L). The level of $p<0.05$ was regarded as significant. The graphs were prepared in GraphPad Prism 5.0.

Results

The leukemia-derived dendritic cells. The purity of leukemic cells before and after the culture was confirmed with CD10, CD19, CD45 and CD79a expression assessed with flow cytometry and was always higher than 95%. After thawing, before the culture, CD40 molecule was expressed in mean 28% of ALL cells (15-

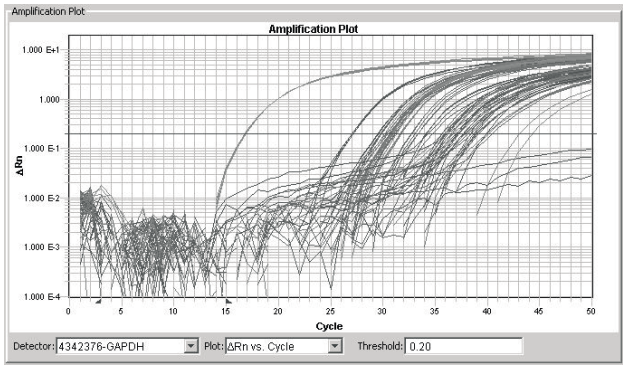

Fig. 1. The example of amplification plot for GAPDH (the internal housekeping gene) from real-time PCR (TaqMan 7900HT).

Table 2 Results: mRNA levels of TAAs by leukemia-derived dendritic cells as compared to unmodified ALL cells and ALL cells cultured with medium (control cells) – median values of $2^{-\Delta\Delta CT}$

Gene symbol	Before the culture (after thawing)	Leukemia-derived dendritic cells (after culture with CD40L+IL-4 +medium) II	Control cells (after culture with medium) III	Statistical analysis		
				I vs II	I vs III	II vs III
PRAME	6061.7	1903.7	243.6	*	**	*
PNPT1	0.189	0.334	0.231	*	-	*
PMPCB	0.306	0.570	0.379	*	-	*
WT1	53.2	452.0	1163.0	*	**	**
HMMR	0.105	0.424	0.045	*	-	**
STEAP3	449.0	264.5	4485.4	-	*	*
BSG	1.168	2.698	1.198	*	-	*
FMOD	56.9	98.4	78.6	-	-	-
BIRC5	0.747	0.630	0.150	-	*	*
PCNA	0.442	0.479	0.307	-	-	-
ERCC1	2.067	4.520	1.712	*	-	*
JUNB	51.280	13.968	15.662	*	*	-
RAF1	1.132	1.486	1.557	-	-	-
BAGE	0.212	1.285	4.375	*	*	**
MAZ	1.082	1.077	1.001	-	-	-

statistical analysis: * $p<0.05$, ** $p<0.01$

40%). As we expected from our previous experiments after 4 days of culture with CD40L/IL-4 ALL cells showed DC morphology and phenotype including characteristic vails and a kidney-shaped nucleus [2, 16]. Flow cytometric analysis demonstrated up-regulation of costimulatory molecules and DC-markers on ALL-cells after CD40 ligation: CD1a, CD11c, CD54, CD80, CD83, CD86 and CD123 (data not shown). Also the mRNA levels for CD1a, CD54, CD80, CD83 and CD86 were higher after the culture with CD40L and IL-4 comparing to the native ALL cells (data not shown). The differences between native ALL cells, ALL-DCs and ALL after culture with medium were statistically significant ($p<0.05$). Additionally, the percentages of the cells with co-expression of DC markers and costimulatory/adhesion molecules and their mRNA levels were higher after the culture with CD40L/IL-4 than after the culture with medium alone ($p<0.05$). Thus upregulation of those molecules can be exclusively caused by CD40 ligation. These results confirmed the possibility for turning leukemic cells into dendritic-like cells.

Expression of tumor associated antigens by ALL-derived DCs. The presence (or absence) of mRNA in ALL specimens for assessed TAAs is presented in Table 1. The mean values of mRNA levels for TAA present in ALL cells before and after the culture are shown in Table 2. From chosen 22 TAA genes 15 were present in at least 10% of ALL cases. The expression of MAZ, FMOD, BIRC5, PCNA and RAF1 in ALL cells did not change after the culture with cytokines. The expression of PRAME, STEAP3 and JUNB was down-regulated in ALL-DCs. The expression of WT1 and BAGE was higher after CD40-activation and culture with medium. The most interesting results concerned PNPT1, PMPCB, HMMR (RHAMM), BSG and ERCC1 antigens which were significantly up-regulated after CD40-ligation but not after the culture with medium alone (Fig. 2.).

Discussion

Since it is unclear whether leukemia-derived dendritic cells maintain to express tumor associated antigens we decided to assess the mRNA levels of the most known TAAs in native ALL cells and ALL-DCs. In our experimental model we used CD40-ligation to convert acute lymphoblastic leukemia cells into dendritic cells. The up-regulation of costimulatory/adhesion molecules noted in flow cytometry and real-time PCR confirmed the dendritic cells-like phenotype of leukemic cells obtained in our experiment. The leukemic origin of DCs was demonstrated by the continuous expression of surface ALL antigens (i.e. CD10, CD19). In other studies the leukemic nature of DC obtained after CD40-ligation was confirmed by detection of chromosomal abnormalities by fluorescence in situ hybridization (FISH) [5, 6]. It is worth to note that our model of immunotherapy does not require genetic manipulation of neoplastic cells. The results of the present study showed maintained expression and even up-regulation of some tumor associated antigens in CD40-activated leukemic cells. In our opinion the higher expression of TAAs on leukemia-derived dendritic cells might enable the immune system to better eradicate minimal residual disease after intensive chemo-, radio- and/or immunotherapy.

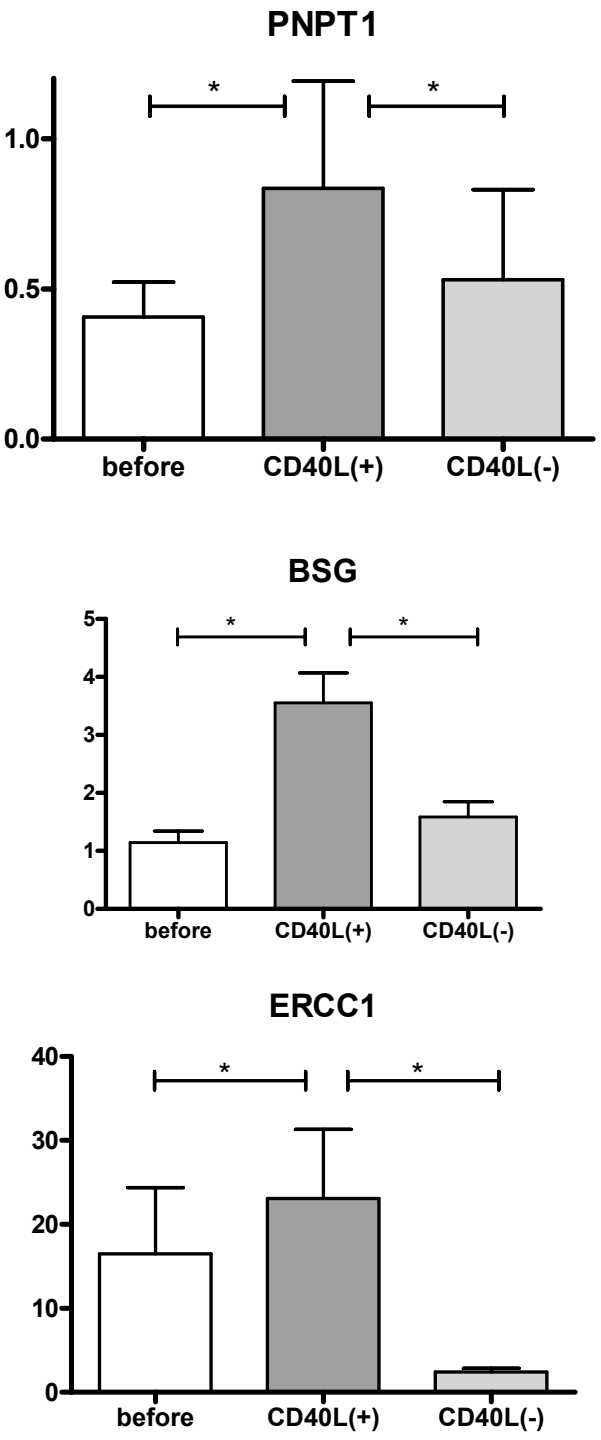

Fig. 2. mRNA levels of chosen TAAs before the culture and after the culture with CD40L/IL-4 (CD40L+) and after the culture with medium alone (CD40L-).

A prerequisite for the development of immunotherapy is the identification and characterization of leukemia-associated antigens. The knowledge about LAAs is very limited and based on very few reliable studies. The expression of some TAA is regarded as related to the prognosis of leukemia. According to Greiner et al. high expression of TAAs was associated with favorable outcome in adult patients with acute myeloid leukemia (AML) [17]. In one experiment preferentially expressed antigen of melanoma (PRAME) overexpression was found in 42% of childhood ALL and was related (but not statistically significant) with good prognosis [18]. In another study, Paydas et al. found a good correlation between PRAME mRNA and remission and/or relapse [19]. In contrast, Spanaki et al. did not find the correlation between PRAME expression and childhood ALL patients survival [20]. In pediatric ALL and AML PRAME is considered as minimal residual disease (MRD) marker, but we showed expression of PRAME in only 10% of ALL specimens [20, 21]. Also Paydas et al. found PRAME in 17% of ALL cases in adults [19]. PRAME overexpression is less frequent in ALL than in AML [18]. This antigen can be the target for cytotoxic T lymphocytes in CML [22]. Basigin (BSG), a novel LAA was found in our experiment in 100% of ALL blasts. We noted upregulation of this gene after CD40 stimulation in ALL cells. The increased expression of BSG was however associated with poor outcome in children with ALL [23]. Data from other laboratories focus on the correlation between the onset of leukemia and TAAs polymorphism e.g. in Chinese population ERCC1 gene polymorphism may be related to the occurence of childhood ALL [24].

RHAMM is one of the most intensively examined TAAs and is expressed by many types of the leukemic cells including ALL, AML and CLL. RHAMM was detected in CLL patients but not in healthy volunteers and was present in 55-90% of CLL and 70% of AML samples [8, 17]. In our study it was expressed in 30% of childhood ALL cases. Fibromodulin and MAZ were also present in majority of CLL cases (55-100%) [8]. In this test PRAME expression was low – 2-20%, and no WT-1, BAGE and MAGE-1 were detected [8]. In our experiment Wilms tumor antigen WT-1, a differentiation gene, was expressed by blasts only in 10%. These results are in opposite to Rezvani et al. who detected WT-1 expression in ALL patients before stem-cell transplantation, SCT [25]. Interestingly, after SCT, the loss of specific anti-WT-1 T lymphocytes was associated with reappearance of WT-1 and molecular relapse. This study supports the role of WT-1 in graft-versus-leukemia effect after SCT. WT-1 among other TAAs can also be used as a MRD marker in pediatric AML [21]. The specific anti-WT1 T-cells were detected in patients with CML and AML but not ALL [26]. According to results of this study and our data the WT1 is one of the future targets of vaccination in myeloid leukemias (AML and CML) but not ALL. For the heat shock 70-kDa protein 4-like (HSPA4L), our results are contrary to the data reported in the literature: HSPA4L was overexpressed in ALL, AML and CML patients [27]. Survivin, an apoptosis inhibitor, was detected in 75% of ALL specimens. It is also

expressed in CLL patients [28]. Interestingly the survivin-specific in vitro-generated T cells recognized CLL cells but not autologous B-cells or dendritic cells [28]. Melanoma antigen family A (MAGE) gene was found by Martinez et al. in a proportion of human leukemias including ALL (37%) and AML but not in our ALL samples [29]. Some authors showed STEAP (a prostate tumor antigen) overexpression in ALL, but in our samples only STEAP3 was expressed in 23%, STEAP1 and STEAP4 were not detected [30]. In contrast to our findings Niemeyer et al. observed frequent expression of SSX-1 and SXX-2 in ALL blasts [9]. ERCC1, upregulated in ALL cells after CD40 stimulation, is considered to be upregulated in childhood ALL de novo cases [31]. According to the authors of this report PCNA and ERCC1 might contribute to development of the disease. The search for additional LAAs with frequent expression in ALL blasts is warranted, allowing the development of future leukemia vaccines.

To date only few important reports concern TAA expression in leukemia-derived dendritic cells. Mayr et al. turned CLL cells into antigen-presenting cells with the use of CD40L viral transduction or CD40L stimulation [13]. Fibromodulin, MDM2, survivin and p53 were chosen as a potential TAAs with high expression in CLL cells but not normal B lymphocytes [32]. Authors of these interesting experiments noted higher numbers of TAA-specific T cells after CD40L viral transduction comparing to the simple CD40L stimulation. The other tests were conducted in AML: dendritic cells generated from AML blasts showed elevated PRAME and RHAMM expression comparing to original AML cells (in our study this phenomenon concerned: RHAMM, PNPT1, PMPCB, BSG and ERCC1). This upregulation also concerned WT-1 and proteinase-3 genes but was present only in a small number of samples [14]. The upregulation of TAA in leukemia-derived dendritic cells can be the possible mode of action of CD40L based cancer vaccines. Data from our investigation stress new features of leukemia-derived dendritic cells and the need for further research in this field. The clinical significance of LAAs up-regulation in ALL-DCs remains unknown.

Autologous ALL-derived DCs might be used as a vaccine potentially eliciting LAA-specific T-cell responses. Three LAAs are the aim of vaccination trials in AML: the proteinase 3-derived peptide (PR1), WT-1 and the RHAMM/CD168 [7, 33]. First results showed safety of this therapeutic approach, the presense of specific immune response and moderate clinical efficacy [34]. Other trial introduced combined vaccine of two LAAs: PR1 and WT1 in leukemic patients [10]. To date the immunogenicity of this combination was demonstrated. Other researchers also postulate the use of polyvalent vaccination [11]. New treatment approaches using CD40-stimulated ALL cells have shown some promising results [35]. However the time point of vaccination is still to be determined [36].

In conclusion, CD40L stimulation leading to the differentiation of leukemic cells into DCs which combine both APC function and expression of tumor associated antigen represents an interesing approach in cancer immunotherapy. From the clinical point of view the upregulation in TAAs expression could offer a useful effect in future immunotherapy. Our results confirm the possibility of turning leukemic cells into dendritic cells and add new aspects to previous knowledge about leukemia-derived dendritic cells.

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