Low expression of costimulatory molecules and mRNA for cytokines are important mechanisms of immunosuppression in acute lymphoblastic leukemia in children?

W. ŁUCZYŃSKI[1], A. STASIAK-BARMUTA[2], E. IŁENDO[3], O. KOVALCHUK[4], M. KRAWCZUK-RYBAK[1], I. MALINOWSKA[5], M. MITURA-LESIUK[6], L. CHYCZEWSKI[4], M. MATYSIAK[5], J. KOWALCZYK[6], R. JAWOROWSKI[3]

[1]Department of Pediatric Oncology, e-mail: vlodek@amb.edu.pl, [2]Flow Cytometry Unit, [3]Department of Cytogenetics, and [4]Department of Clinical Molecular Medicine, Medical University of Bialystok, Bialystok, Poland; [5]Department of Pediatrics, Hematology and Oncology, Medical University of Warszawa, and [6]Department of Pediatric Hematology and Oncology, Medical University of Lublin, Poland

Received January 10, 2006

Mechanisms leading blasts of acute lymphoblastic leukemia to escape from immune surveillance are still unknown. Only few reports showed that ALL cells are inefficient antigen presenting cells. The aim of the study was to assess expression of critical costimulatory/adhesion molecules and mRNA for main pro- and anti-inflammatory cytokines in ALL cells. Children with B-cell precursor ALL (n=20) were prospectively enrolled into the study. Expression of costimulatory/adhesion molecules (CD1a, CD11c, CD40, CD54, CD80, CD83, CD86, CD123, HLA class I and II) was assessed by flow cytometry and mRNA for cytokines (IFN-γ, IL-10, IL-4, TGF-β) – with real-time PCR.

Results: 1) high expression was observed for HLA I and II class, moderate for CD40, CD83, CD86 and low or no expression for CD80, CD54, CD1a, CD11c and CD123; 2) we found expression of mRNA for IFN-γ, IL-10, IL-4 and TGF-β in blasts cells (but not in all specimens). We noted relatively lower expression of all assessed cytokines comparing to T-cells obtained from healthy donors but interestingly expression for IL-10 was higher in normal B-cells than in blast cells, and IFN-γ and IL-4 were not found in normal B-cells.

In summary we suggest that ALL-blasts present low expression of costimulatory/adhesion molecules and mRNA for cytokines and this probably contribute to the absence of host T-cells stimulation to immune response.

Key words: acute lymphoblastic leukemia, children, cytokines, flow cytometry, real-time PCR, costimulatory molecules

Interactions between the cancer and host cells are extensively examined for many years. Recently mechanisms of immunosuppression caused by neoplastic cells are of interest. The aim of this field of research is to introduce and develop immunotherapy in patients suffering of cancer. The most frequent neoplastic disease in children is acute lymphoblastic leukemia (ALL). Mechanisms which ALL blasts escape from immune surveillance are still unknown. To date only few reports showed that ALL cells are inefficient antigen presenting cells.

Expression of costimulatory molecules of B7 family (CD80, CD86) is essential for induction of T-cell response [1]. Antigen presentation in the absence of costimulation induces anergy and is one of the mechanisms in which neoplastic cells probably escape from immune recognition. Previously we have shown Th_2 lymphocytes (IL-4, TGF-β) predominance in leukemias and IFN-γ deficiency in lymphomas in children [2]. Abnormalities of T cell function play an important role in defective immune response against leukemia cells, but it is believed that lack of antigen presentation by ALL cells is the main mechanism of this phenomenon. Some authors suggest that immune dysfunction concomitant with leukemia appearance is caused not only by lack of costimulation but also by release of immunosuppressive cytokines, such as IL-10 or TGF-β [3]. Few reports demonstrated the cytokine-producing ability of malignant B cells [4]. Recent studies have shown that expression of cytokines/cytokine receptors in leukemic cells can be associated with survival rate [5].

Abbreviations: ALL – acute lymphoblastic leukemia, AML – acute myeloid leukemia, APC – antigen presenting cell, BCP – B-cell precursor, CLL – chronic lymphocytic leukemia, CML – chronic myeloid leukemia

Previously we have studied the role of T-lymphocytes and monocytes in immunosuppression in children with cancer. This time we wanted to analyze immunologic behaviour of neoplastic cells. The aim of this study was to assess expression of critical costimulatory/adhesion molecules and mRNA for main pro- and anti-inflammatory cytokines in ALL cells.

Material and methods

Patients. Children with BCP-ALL were prospectively enrolled into the study N=20 (departments of pediatric hematology and oncology in Bialystok, Warszawa and Lublin). Bone marrow (5 ml) or peripheral blood (white blood count $>10x10^6$/l, neoplastic cells >50%) were collected at diagnosis before any treatment. 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) solution and immediately analyzed with flow cytometry.

Flow cytometry. Following fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- or peridinin chlorophyll protein (PerCP)-conjugated monoclonal antibodies (and isotype controls) were used: CD1a, CD10, CD11c, CD19, CD40, CD45, CD54, CD80, CD83, CD86, CD123, HLA class I, HLA-DR (all purchased from Beckton Dickinson, USA). A total of 10^5 cells were incubated with monoclonal antibodies for 30 minutes at 4 °C, washed twice and analyzed on a EPICS XL (Coulter) flow cytometer. Percentages of positive cells were calculated. Leukemia cells were separated with the use of CD45, CD10 and CD19 antigens expression.

Real time PCR. $CD4^+$, $CD8^+$, $CD19^+$ (from healthy volunteers) and $CD10^+$ (only in $CD10^+$ ALL cases) cells were isolated from whole peripheral blood by immunomagnetic bead separation (Dynabeads, Dynal Biotech, Oslo, Norway). mRNA was isolated from sorted lymphocyte subpopulations using Dynabeads mRNA Direct Micro Kit (Dynal) according to the producer instructions. First strand cDNA was synthesized using random hexamers as primer and High Capacity cDNA Archive Kit by Applied Biosystems. Four different cytokine mRNAs level (IFN-γ, IL-10, IL-4, TGF-β_1) were determined by real-time PCR technique with the TaqMan chemistry using ready-to-use Assays-on-Demand Gene Expression Products by Applied Biosystems. To normalize the amount of expressed cytokine mRNAs the internal housekeeping gene GAPDH was used and each complementary DNA (cDNA) product was tested in triplex for each of four cytokines mRNA and GAPDH mRNA. To calculate our data we used Comparative C_t method for relative quantification ($\Delta\Delta C_t$ method). As a calibrator sample we used Total Raji RNA by Applied Biosystems which was processed in the same way as the test samples.

The institutional review board approved this study and informed consent was obtained from patients and their parents.

Results and discussion

Flow cytometry. Results obtained from flow cytometry are summarized in Table 1 and Figure 1. High expression for HLA I and II class was observed, moderate for: CD40, CD83, CD86 and low or no expression for CD80, CD54, CD1a, CD11c and CD123.

Table 1. Median percentages of ALL cells with costimulatory and adhesion molecules expression

Molecule	Median percentage of positive cells
HLA I class$^+$	79.0%
HLA II class$^+$	64.0%
$CD40^+$	24.0%
$CD83^+$	18.0%
$CD1a^+$	6.0%
$CD54^+$	7.0%
$CD80^+$	5.0%
$CD86^+$	14.0%
$CD123^+$	0.2%
$CD11c^+$	0.2%
$CD80^+CD86^+$	5.0%

Similarly to our results in a few another studies ALL cells were strongly positive for HLA class I and II, moderately for CD40 (28%), CD83 (11–13%), CD86 (26–41%) and negative for CD1a and CD80 [6–8]. In HAINING et al's study ALL cells were highly positive for MHC I and II class, but mostly negative for CD80, CD86 (expression in 5% and 14%, respectively in our study) and CD54 (7% in our group) [9]. In contrast to ALL, AML cells express CD86 in 100% so CIGNETTI et al regard expression of CD80 and CD83 on neoplastic cells as dendritic cells markers [10, 11]. ALL blasts with translocation (9;22) express CD80 molecule on their surface but only in 0.49 – 9% [12]. In CIGNETTI et al's opinion malignant cells have low immunogenicity due in part to low expression of costimulatory molecules (CD80, CD86). They report successfull differentiation of blast cells into dendritic cells with CD40 ligation [7].

It is known for some time that interaction of CD28 (on T-cells) and CD86 (on APCs) favors the generation of Th_2 response, but CD28/CD80 interaction is favorable to Th_1 expansion [13]. This could explain our previous results – expression of CD86 but not CD80 on ALL cells promotes Th_2 predominance [14].

Real-time PCR. Results obtained from real-time PCR are presented in Figure 2. We found the expression of mRNA for IFN-γ, IL-10, IL-4 and TGF-β in blasts cells (but not in all specimens). We noted relatively lower expression of all assessed cytokines comparing to T-cells obtained from healthy donors (statistical analysis was not performed because of small number of samples) but interestingly expression for

IL-10 was higher in normal B-cells than in blast cells, and IFN-γ and IL-4 were not found in normal B-cells.

The autocrine production of cytokines by leukemic cells may have a role in the pathogenesis of the disease. AMILLS et al provide the evidence that B cells infected with bovine leukemia virus express specific cytokine mRNAs e.g. TNF-α, TGF-β_1, IL-10 and IL-6 [15]. SCHULZ et al found the expression of mRNA for IL-10 and TNF-α in ALL, AML and CML blasts [16]. High expression of immunosuppressive cytokine (IL-10) and low of proinflammatory one (TNF-α) were related with the failure in response to immunotherapy (donor lymphocyte infusions). Surprisingly, in our study, mRNA levels of IL-10 in ALL cells were lower than in normal B-cells ($CD19^+$). In comparison according to WU et al expression of IL-10 and IFN-γ was higher in BCP than in T-lineage blasts [5]. We found mRNA for IL-4 in a few blast cells, but not in normal B-lymphocytes. B-CLL cells secreted more IL-4 than $CD8^+$ T cells and this production was associated with increasing cell numbers [17]. Addition of anti-IL-4 increased the apoptotic rate of B-CLL cells, which suggests some role of this cytokine in cell cycle in this disease. Also ALL blasts with translocation (9;22) have detectable levels of IL-4 (determined by flow cytometry) [12].

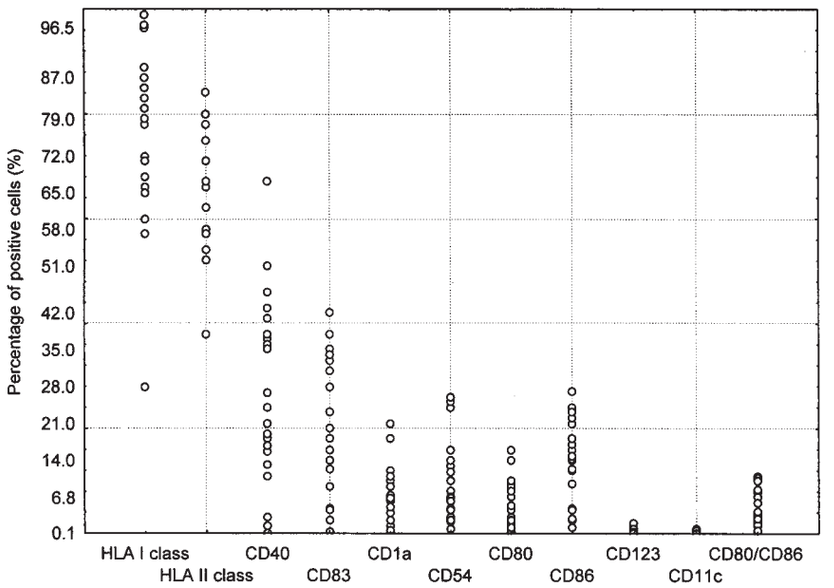

Figure 1. Expression of costimulatory and adhesion molecules on ALL cells assessed in flow cytometry.

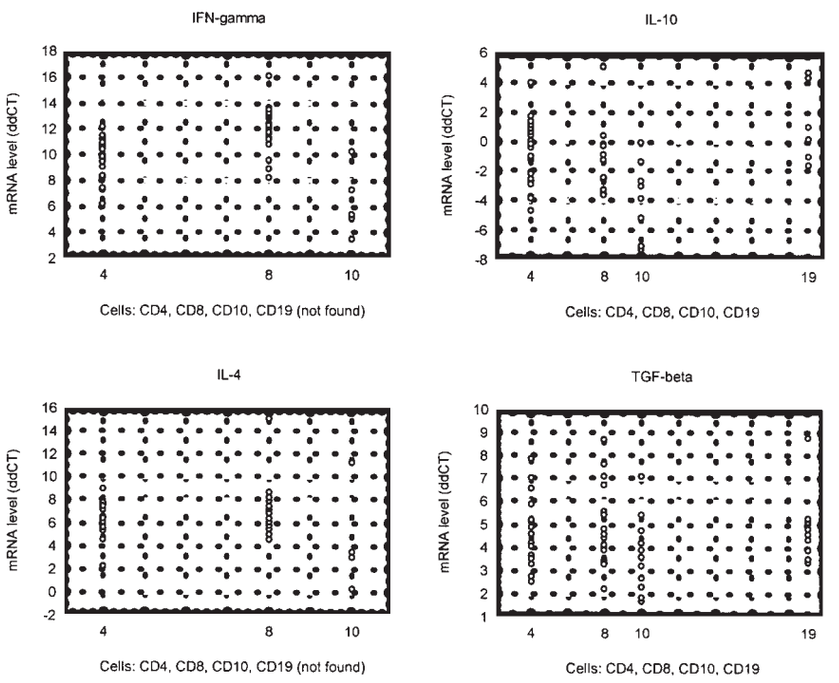

Figure 2. mRNA levels for pro- and anti-inflammatory cytokines in T-cells (CD4, CD8), B-cells (CD19) and ALL-cells (CD10).

The analysis of the gene expression was performed in leukemic cells from children with relapsed ALL [4]. Authors confirmed presence of transcripts for IL-10 and IFN-γ but opposite to our findings, IL-4 was not found. MHC class I and II were detectable in all specimens. Neoplastic cells were all negative for CD80 but in 14% positive for CD86, median expression for CD54 and CD40 was low (3%). There was no correlation between event-free survival and expression level of any assessed molecule or cytokine level. In WU et al's experiments lymphoblasts from children with ALL at first recurrence exhibited high expression of IL-10 and TGF-β and moderate expression of IFN-γ. The expression of IL-10 mRNA was higher than in initial ALL blasts but TGF-β – lower [5]. Expression of TGF-β and IL-10 (the most potent immunosuppressive agents) in leukemia cell line correlated with tumor progression and was responsible for affected cytolytic ac-

tivity of T cells in mice treated with the vaccine [18]. Some authors suggest that ALL blasts did not produce IL-10 [6]. B-CLL cells produced low amounts of IL-10 even after stimulation [1]. Despite presence of IL-10 in blast cells the definite function of IL-10 in ALL pathogenesis requires further investigation.

AML cells secreted surprisingly proinflammatory cytokines IFN-γ, IL-12 (Th_1 profile) and also IL-10 [19]. This expression was not correlated with prolonged survival.

In summary we suggest that ALL-blasts present low expression of costimulatory/adhesion molecules and mRNA for cytokines and probably this is why they can not stimulate host T-cells to immune response. Transfection of costimulatory molecules could render non-immunogenic malignant cells immunogenic [20]. Further studies are thus warranted.

References

[1] HOOGEDOORN M, WOLBERS JO, SMIT WM, SCHAAFSMA MR, BARGE RMY et al. Generation of B-cell chronic lymphocytic leukemia (B-CLL)-reactive T-cell lines and clones from HLA class I-matched donors using modified B-CLL cells as stimulators: implications for adoptive immunotherapy. Leukemia 2004; 18: 1278–1287.

[2] LUCZYNSKI W, KOVALCHUK O, KRAWCZUK-RYBAK M, MITURA-LESIUK M, MALINOWSKA I et al. Lower expression of mRNA for interferon-gamma in T helper cells of children with newly diagnosed lymphomas. Folia Histoch Cytobiol 2005; 43: 169–171.

[3] ROMANO C, FANIS UD, SELLITO A, MORA LD, CHIURAZZI F et al. Effects of preactivated autologous T lymphocytes on CD80, CD86 and CD95 expression by chronic lymphocytic leukemia B cells. Leuk Lymphoma 2003; 44: 1963–1971.

[4] KEBELMANN-BETZING C, KORNER G, BADIALI L, BUCHWALD D, MORICKE A et al. Characterization of cytokine, growth factor receptor, costimulatory and adhesion molecule expression patterns of bone marrow blasts in relapsed childhood B cell precursor ALL. Cytokine 2001; 13: 39–50.

[5] WU S, GESSNER R, STACKELBERG AV, KIRCHNER R, HENZE G et al. Cytokine/cytokine receptor gene expression in childhood acute lymphoblastic leukemia. Cancer 2005; 103: 1054–1063.

[6] D'AMICO G, BUGARIN MV, BIANCHI G, PIROVANO G, BONAMINO M et al. Activation of BCP-ALL cells through CD40 generates IL-10 producing, IL-12 defective APC inducing allogeneic T-cell anergy. Blood 2004; 104: 744–751.

[7] CIGNETTI A, BRYANT E, ALLIONE B, VITALE A, FOA R et al. CD34+ acute myeloid and lymphoid leukemic blasts can be induced to differentiate into dendritic cells. Blood 1999; 94: 2048–2055.

[8] TODISCO E, GAIPA G, BIAGI E, BONAMINO M, GRAMIGNA R et al. CD40 ligand-stimulated B cell precursor leukemic cells elicit interferon-gamma production by autologous bone marrow T cells in childhood acute lymphoblastic leukemia. Leukemia 2002; 16: 2046–2054.

[9] HAINING WN, CARDOSO AA, KECZKEMETHY HL, FLEMING M, NEUBER D et al. Failure to define window of time for autologous tumor vaccination in patients with newly diagnosed or relapsed acute lymphoblastic leukemia. Exp Hematol 2005; 33: 286–294.

[10] CIGNETTI A, VALLARIO A, ROATO I, CIRCOSTA P, ALLIONE B et al. Leukemia-derived immature dendritic cells differentiate into functionally competent mature dendritic cells that efficiently stimulate T cells responses. J Immunol 2004; 173: 2855–2865.

[11] URBANIAK-KUJDA D, WOLOWIEC D, TOMASZEWSKA-TOPORSKA B, JAZWIEC BE, FRYDECKA I et al. The influence of short culture of leukemic blasts on expression of co-stimulatory molecules and the ability to induce the proliferatin of autologous T lymphocytes. Adv Clin Exp Med 2004; 13: 567–573.

[12] LEE J, SAIT SN, WETZLER M. Characterisation of dendritic-like cells derived from t(9;22) acute lymphoblastic leukemia blasts. Int Immunol 2004; 16: 1377–1389.

[13] YOTNDA P, MINTZ P, GRIGARIADOU K, LEMONNIER F, VILMER E et al. Analysis of T-cell defects in the specific immune response against acute lymphoblastic leukemia cells. Exp Hematol 1999; 27: 1375–1383.

[14] LUCZYNSKI W, STASIAK-BARMUTA A, KRAWCZUK-RYBAK M, MALINOWSKA I. Assessment of chosen costimulatory, adhesion, activatory molecules and cytokines of Th_1/Th_2 balance in acute lymphoblastic leukaemia in children. Arch Immun Ther Exp 2005; 53: 357–363.

[15] AMILLS M, NORIMINE J, OLMSTEAD CA, LEWIN HA. Cytokine mRNA expression in B cells from bovine leukemia virus-infected cattle with persistent lymphocytosis. Cytokine 2004; 28: 25–28.

[16] SCHULZ U, MUNKER R, ERTL B, HOLLER E, KOLB HJ. Different types of human leukemias express the message for TNF-alpha and interleukin-10. Eur J Med Res 2001; 6: 359–363.

[17] KAY NE, HAN L, BONE N, WILLIAMS G. Interleukin 4 content in chronic lymphocytic leukaemia (CLL) B cells and blood $CD8^+$ T cells from B-CLL patients: impact on clonal B-cell apoptosis. Br J Haematol 2001; 112: 760–767.

[18] HSIEH CL, CHEN DS, HWANG LH. Tumor-induced immunosuppression: a barrier to immunotherapy of large tumors by cytokine-secreting tumor vaccine. Hum Gene Ther 2000; 11: 681–692.

[19] PANOSKALTSIS N, REID CD, KNIGHT SC. Quantification and cytokine production of circulating lymphoid and myeloid cells in acute myelogenous leukaemia. Leukemia 2003; 17: 716–730.

[20] MOHTY M, ISNARDON D, CHARBONNIER A, LAFAGE-POCHITALOFF M, MERLIN M et al. Generation of potent Th1 responses from patients with lymphoid malignancies after differentiation of B lymphocytes into dendritic cells. Int Immunol 2002; 14: 741–750.