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Reduction in Proportion of Senescent CD8+ T Lymphocytes During Chemotherapy of Children with Acute Lymphoblastic Leukemia

VILMA RUTKAUSKAITĖ[1,2], LINA RAGELIENĖ[2,3], RĖDA MATUZEVIČIENĖ[4,5], KATAŽINA SINKEVIČ[4], MYKOLAS MAURICAS[6] and DAINIUS CHARACIEJUS[1,6]

[1]*Department of Pathology, Forensic Medicine and Pharmacology, Faculty of Medicine, Vilnius University, Vilnius, Lithuania;*
[2]*Center for Pediatric Oncology and Hematology, Children's Hospital, Affiliate of Vilnius University Hospital Santariškių Klinikos, Vilnius, Lithuania;*
[3]*Clinic of Children's Diseases, Faculty of Medicine, Vilniaus University, Vilnius, Lithuania;*
[4]*Center of Laboratory Medicine, Vilnius University Hospital Santariškių Klinikos, Vilnius, Lithuania;*
[5]*Department of Physiology, Biochemistry, Microbiology and Laboratory Medicine, Faculty of Medicine, Vilnius University, Vilnius, Lithuania;*
[6]*Department of Immunology, Center for Innovative Medicine, Vilnius, Lithuania*

Abstract. *Aim: To evaluate quantitative changes in B, NK and T lymphocyte subsets in peripheral blood of children with acute lymphoblastic leukemia (ALL) undergoing chemotherapy. Patients and Methods: Children with ALL were treated according to NOPHO ALL 2008 protocol. Levels of B lymphocytes ($CD19^+$), NK cells ($CD3^-CD56^+$) and subsets of T lymphocytes ($CD3^+CD4^+$, $CD4^+CD25^+Foxp3^+$, $CD3^+CD8^+$, $CD3^+CD8^+CD57^+$, $CD3^+CD8^+CD57^-$) in peripheral blood were analyzed by flow cytometry prior and during treatment with cytotoxic drugs. Results: Immunological analyses were performed in 25 children with ALL. Levels of B and NK lymphocytes decreased continuously during chemotherapy. In contrast, levels of most T lymphocyte subsets decreased only transiently and returned to pretreatment levels by days 78 to 85. The only T lymphocyte subset that did not return to the pretreatment level contained senescent $CD3^+CD8^+CD57^+$ lymphocytes. Conclusion: Immunomodulating action of chemotherapy in children with ALL results in reduction of proportion of senescent $CD8^+$ T lymphocytes.*

Correspondence to: Professor Dainius Characiejus, Faculty of Medicine, Vilnius University, M.K. Čiurlionio 21, LT-03101 Vilnius, Lithuania. Tel: +370 52398728, Fax: +370 52398705, e-mail: dainius.characiejus@gmail.com

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Accumulating evidence indicates that antitumor activities of chemotherapy rely on stimulation of the immune system. One of the proposed mechanisms of immunomodulating action of cytotoxic agents is their influence on the immune homeostasis by induction of transient lymphodepletion followed by rebound replenishment of immune cell pools (1). However, it is not clear what changes in populations of immune cells are associated with antitumor effect(s) of chemotherapy.

Acute lymphoblastic leukemia (ALL) in children is a disease that is effectively cured by the best contemporary protocols (2). Analysis of immune changes in children with ALL undergoing effective chemotherapy would provide an insight as to which of the immune parameters might be related with antitumor effect(s). In this study, we analyzed changes in B lymphocytes, NK cells and subsets of T lymphocytes in children with ALL during treatment according to the NOPHO ALL 2008 protocol (2). Among T lymphocyte subsets, we were especially interested in changes of Foxp3-expressing T-regulatory lymphocytes and CD57 antigen-positive and -negative $CD8^+$ lymphocytes.

Patients and Methods

Patients and treatment. The study was performed at the Center for Pediatric Oncology and Hematology, Children's Hospital, Affiliate of Vilnius University Hospital Santariškių Klinikos, Vilnius, Lithuania. The study protocol was approved by Vilnius Regional Biomedical Research Ethics Committee. All study participants or their legal guardians provided written informed consent prior to study enrollment.

Patients diagnosed with Philadelphia chromosome-negative B-cell precursor or T-lineage ALL and scheduled chemotherapy were included in this study. Criteria for exclusion were prior anticancer treatment (including steroids) and diseases that might influence peripheral blood lymphocyte levels (including myelodysplastic syndrome).

All patients were treated according to the NOPHO ALL 2008 protocol (2). Induction treatment consisted of daily oral prednisolone or intravenous methylprednisolone (60 mg/m^2/dose) (oral or intravenous dexamethasone 10 mg/m^2/dose for T-lineage ALL) on days 1-29, then tapered during 9 days and discontinued, weekly intravenous vincristine (2 mg/m^2/dose, not exceeding 2.5 mg/dose), 2 doses of intravenous doxorubicin (40 mg/m^2/dose) on days 1 and 22, 1 dose of intramuscular PEG-asparaginase (1,000 IU/m^2/dose) on day 30 and intrathecal methotrexate on days 1, 8, 15 and 29 in age-adjusted doses (8-12 mg/dose). One patient with Down syndrome received doxorubicin only on day 22 and additional dose of PEG-asparaginase on day 8.

Consolidation treatment of ALL patients differs according to risk groups (standard, intermediate or high). There were no high-risk patients in our study. Therefore, consolidation treatment was given as for standard and intermediate risk groups. This stage of treatment consisted of daily oral mercaptopurine (25 mg/m^2/dose) from day 30 to day 85, intravenous methotrexate (5 g/m^2/dose) on days 36, 57 and 78, intramuscular PEG-asparaginase (1,000 IU/m^2/dose) on days 43, 57, 71 and 85, intravenous vincristine (2 mg/m^2, but not exceeding 2.5 mg/ dose) on days 43 and 64 and intrathecal methotrexate on days 36, 57 and 78 in age-adjusted doses (8-12 mg/dose). For one patient with Down syndrome, doses of intravenous methotrexate were reduced to 2 g/m^2/dose. Patients with central nervous system involvement received intrathecal methotrexate, cytarabine and methylprednisolone, according to NOPHO ALL 2008 protocol (2).

Morphologic remission was defined as <5% leukemic blasts in the bone marrow. Minimal residual disease (MRD) was assessed by flow cytometry quantitation of residual leukemia for patients with B-cell precursor phenotype and by polymerase chain reaction (PCR) assessment for patients with T-lineage phenotype.

FACS analysis of peripheral blood lymphocyte subsets. Peripheral blood samples for flow cytometry analysis were collected prior to treatment, at the end of induction phase (days 29 to 36) and at the end of consolidation phase (days 78 to 85). Lymphocytes were stained with the following combinations of fluorochrome-conjugated monoclonal antibodies: CD45-V500/CD4-V450/CD57-FITC/Foxp3-PE/CD25-APC/CD3-Pe-Cy7/CD8-APC-Cy7 and CD45-V500/CD4-V450/CD57-FITC/CD56-PE/CD19-PerCP-Cy5.5/CD27-APC/CD3-Pe-Cy7/CD8-APC-Cy7 (Becton Dickinson, San Jose, CA, USA). The samples were analyzed using FACSCanto II flow cytometer and FACS Diva version 6.1.3. software (Becton Dickinson).

Absolute counts of white blood cells were measured on a Sysmex XT-4000i hematology analyzer (Sysmex Corporation, Kobe, Japan). Peripheral blood smears were stained with Giemsa/May–Grünwald stain and percentages of all leukocyte populations, including leukemic blasts, were counted during evaluation under microscope. The absolute counts of circulating non-leukemic lymphocytes per mm^3 were calculated by multiplying the percentage of non-leukemic lymphocytes in the blood smear by the absolute number of white blood cells. These absolute numbers of circulating non-leukemic lymphocytes were used to calculate the absolute counts of lymphocyte subsets.

Table I. *Patients' characteristics*.

	n=25
Age months, median (range)	53 (18-169)
Male/female	13/12
Phenotype:	
Pre-B ALL	24
T-lineage ALL	1
Standard risk group	18
Intermediate risk group	7
Positive MRD on day 29	3
Down syndrome	1
T-lineage ALL	1
t(1;19)	1
ic21amp	1
CNS1	23
CNS2	1
CNS3	1

CNS1, No blast cells in cerebrospinal fluid; CNS2, <5 white blood cells/μl cerebrospinal fluid with blast cells; CNS3, ≥5 white blood cells/μl cerebrospinal fluid with blast cells, or signs of central nervous system involvement. CNS, central nervous system; ALL, acute lymphoblastic leukemia; MRD, minimal residual disease.

Statistics. Statistical analysis was performed using SPSS software version 20.0 (Armonk, NY, USA). The differences between lymphocyte subset values at different time points were evaluated using the Wilcoxon signed-rank test. The level of significance was set at p=0.05.

Results

Twenty five children with ALL were enrolled in the study from September 2013 to January 2016. Demographic and clinical characteristics of patients are shown in Table I. Morphologic remission was achieved in all 25 patients at the end of induction phase of treatment, while MRD was present (0.1-5%) in the bone marrow of 3 patients. All patients were MRD-negative at the end of consolidation phase of treatment.

Changes in absolute counts of B ($CD19^+$) and NK ($CD3^-CD56^+$) lymphocytes during chemotherapy of children with ALL are shown in Figure 1. Absolute numbers of $CD19^+$ lymphocytes decreased from 1,463 cells/μl prior to treatment to 492 cells/μl at the end of induction phase and to 82 cells/μl at the end of consolidation phase. $CD3^-CD56^+$ lymphocyte counts decreased from 385 cells/μl prior to treatment to 64 cells/μl at the end of induction phase. Similar values of $CD3^-CD56^+$ lymphocytes (67 cells/μl) remained at the end of consolidation phase.

Pretreatment $CD3^+CD4^+$ lymphocyte counts of 1,737 cells/μl and $CD3^+CD8^+$ lymphocyte counts of 1,424 cells/μl decreased to 736 cells/μl and 645 cells/μl, respectively, at the end of induction phase of treatment. Despite continuation of

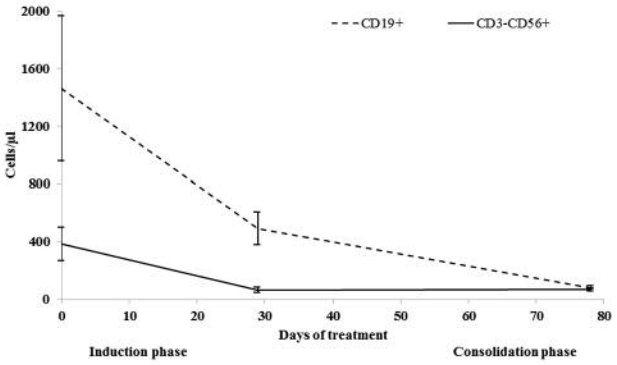

Figure 1. *Changes in levels of B (CD19+) and NK (CD3−CD56+) lymphocytes in peripheral blood during chemotherapy of children with ALL. Peripheral blood samples were collected prior to treatment, at the end of induction phase (days 29-36) and at the end of consolidation phase (days 78-85). Lymphocytes were analyzed by flow cytometry. The decrease in B lymphocyte counts is statistically significant at the end of induction phase and at the end of consolidation phase compared to pretreatment value (p=0.006 and p=0.001, respectively). Decrease in NK lymphocyte counts is statistically significant at the end of induction phase and at the end of consolidation phase compared to pretreatment value (p=0.001). Values are given as means±SEM.*

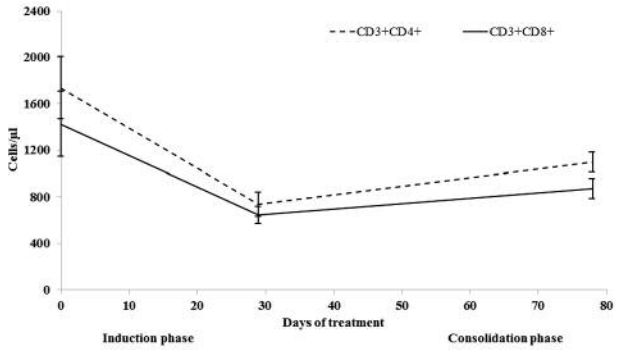

Figure 2. *Changes in CD3+CD4+ and CD3+CD8+ lymphocyte counts in peripheral blood during chemotherapy of children with ALL. Peripheral blood samples were collected prior to treatment, at the end of induction phase (days 29-36) and at the end of consolidation phase (days 78-85). Lymphocytes were analyzed by flow cytometry. Decrease in CD3+CD4+ and CD3+CD8+ lymphocyte counts at the end of induction phase is statistically significant compared to pretreatment values (p=0.001 and p=0.002, respectively). Values are given as means±SEM.*

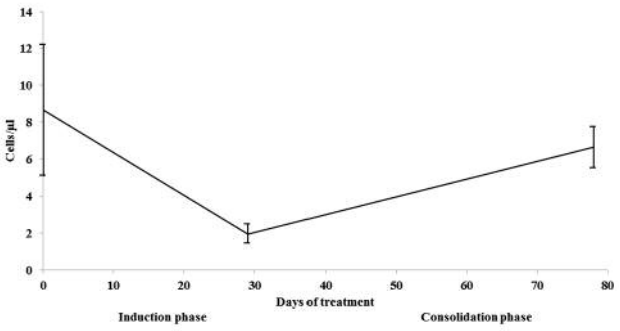

Figure 3. *Changes in T-regulatory (CD4+CD25+Foxp3+) lymphocyte counts in peripheral blood during chemotherapy of children with ALL. Peripheral blood samples were collected prior to treatment, at the end of induction phase (days 29-36) and at the end of consolidation phase (days 78-85). Lymphocytes were analyzed by flow cytometry. Decrease in T-regulatory lymphocyte counts is statistically significant at the end of induction phase compared to pretreatment values (p=0.003). Values are given as means±SEM.*

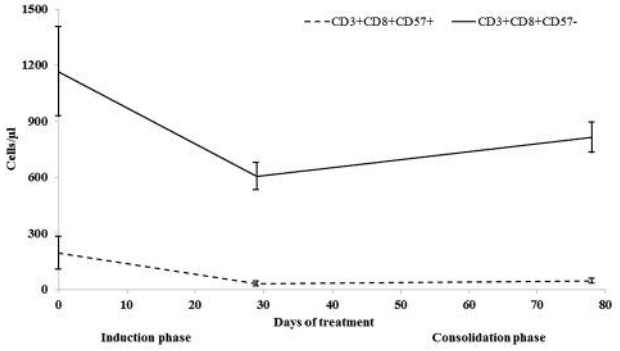

Figure 4. *Changes in CD+CD8+CD57− and CD3+CD8+CD57+ lymphocyte counts in peripheral blood during chemotherapy of children with ALL. Peripheral blood samples were collected prior to treatment, at the end of induction phase (days 29-36) and at the end of consolidation phase (days 78-85). Lymphocytes were analyzed by flow cytometry. Decrease in CD3+CD8+CD57− lymphocyte counts is statistically significant at the end of induction phase compared to pretreatment value (p=0.005). Decrease in CD3+CD8+CD57+ lymphocyte counts is statistically significant at the end of induction phase and at the end of consolidation phase compared to pretreatment value (p=0.001 and p=0.006, respectively). Values are given as means±SEM.*

cytotoxic treatment, significantly reduced levels of both $CD3^+CD4^+$ and $CD3^+CD8^+$ lymphocytes started recovering during the consolidation phase and reached 1,096 cells/µl and 872 cells/µl, respectively (Figure 2).

Counts of T-regulatory ($CD4^+CD25^+FoxP3^+$) lymphocytes decreased from 9 cells/µl prior to treatment to 2 cells/µl at the end of induction phase and then increased, reaching 7 cells/µl at the end of consolidation phase (Figure 3).

Changes in $CD3^+CD8^+CD57^-$ and $CD3^+CD8^+CD57^+$ lymphocyte counts in peripheral blood during treatment are

shown in Figure 4. $CD3^+CD8^+CD57^-$ lymphocyte counts decreased from 1,165 cells/μl prior to reatment to 605 cells/μl at the end of the induction phase. At the end of consolodation phase, the count of $CD3^+CD8^+CD57^-$ lymphocytes reached 812 cells/μl. $CD3^+CD8^+CD57^+$ lymphocyte counts decreased from 196 cells/μl prior to treatment to 30 cells/μl at the end of inducton phase. At the end of consolidation phase, the $CD3^+CD8^+CD57^+$ lymphocyte count was 46 cells/μl. The proportion of senescent $CD3^+CD8^+CD57^+$ lymphocytes in $CD3^+CD8^+$ subset decreased from 11±2.5% prior to treatment to 5±1.3% at the end of consolidation phase (mean±SEM, p=0.001).

Discussion

Our results do not seem to support the possibility that NK lymphocytes are important in antitumor effect(s) of cytotoxic drugs in children with ALL as their levels decreased continuosly during treatment. In regard to B lymphocytes, a subset of B cells, identified as B-regulatory cells, have recently emerged as major contributors to the pathogenesis of autoimmune and neoplastic disorders (3). Similarly to NK cells, levels of B lymphocytes decreased continuously during treatment. Thus, the premise that elimination of B-regulatory lymphocytes by cytotoxic drugs is important in antitumor effect cannot be excluded.

The ability of T-regulatory lymphocytes to facilitate evasion of the immune system by cancerous cells is well-known (4). However, in our study, pretreatment levels of T-regulatory lymphocytes in peripheral blood were relatively low (only 9 cells/μl) and decreased only transiently during treatment. Thus, it is unlikely that antitumor effect(s) of chemotherapy in children with ALL depends on the reduction of T-regulatory lymphocyte levels.

Long-term survival is achieved in more than 80-90% of children with ALL, whereas only 30-40% of adults with this disease are still alive 5 years after diagnosis. This difference can be partially explained by higher incidence of T-lineage ALL and more *MLL* gene rearrangements in older patients (2). However, advanced age remains a dominant predictor of poor clinical outcome for nearly all cancers. It has been shown, in a study of patients with glioblastoma multiforme and in a rodent model of intracranial glioma, that levels and function of newly produced $CD8^+$ T cells critically influence age-dependent cancer mortality (5).

One of the most prominent features of senescence of the immune system is loosing of CD28 and acquisition of CD57 expression by $CD8^+$ T lymphocytes (6). Essentially, all T cells from newborns express CD28 (thus, they are negative for CD57). Young adults have approximately 30% $CD28^-CD57^+$ cells, whereas individuals over 80 years old have over 60% $CD28^-CD57^+$ cells among $CD8^+$ T lymphocytes (7).

In melanoma patients, the expression of CD57 antigen by $CD8^+$ T lymphocytes has been associated with effector functions (8). However, in a number of other human cancers, $CD8^+CD57^+$ T lymphocytes seem unable to inhibit the growth of malignant cells and may even dampen immune responses against tumor-associated antigens, perhaps by competing for resources, such as cytokines or nutrients (9). Increased peripheral blood proportions of $CD57^+$ T lymphocytes have been shown to be associated with poor prognosis in patients with advanced renal cell carcinoma (10) and advanced gastric carcinoma (11).

In our study, children with ALL had a mean of only 11% $CD3^+CD8^+CD57^+$ cells among $CD3^+CD8^+$ lymphocytes prior to treatment and this percentage dropped to 5% during cytotoxic therapy. Interestingly, chemotherapy for breast cancer, which is well-known to be far less effective than for childhood ALL failed to reduce the levels of senescent $CD8^+$ T cells (12). Thus, it can be hypothesized that the high efficacy of chemotherapy in children with ALL may depend on relatively low pretreatment levels of senescent $CD3^+CD8^+CD57^+$ lymphocytes and their reduction during treatment.

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