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Cytokines and soluble HLA-G levels in bone marrow stroma and their association with the survival rate of patients exhibiting childhood T-cell acute lymphoblastic leukemia

Renata dos Santos Almeida[a], Alessandra Maria de Luna Ramos[b], Carlos Feitosa Luna[c], Francisco Pedrosa[b], Eduardo Antônio Donadi[d], Norma Lucena-Silva[a,b,*]

[a] Laboratory of Immunogenetics, Department of Immunology, Aggeu Magalhães Research Center, Oswaldo Cruz Foundation, Recife, PE, Brazil
[b] Pediatric Oncology Service, IMIP Hospital, Recife, PE, Brazil
[c] Department of Collective Health, Aggeu Magalhães Research Center, Oswaldo Cruz Foundation, Recife, PE, Brazil
[d] Division of Clinical Immunology, Department of Medicine, School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil

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ABSTRACT

Leukemic cells can induce defective expression of soluble factors and change marrow cytokine profile, leading to aberrant cell signaling, cell fixation and cell proliferation in bone marrow. T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive disorder which accounts for 15% of pediatric ALL. To evaluate the contribution of immunological factors on T-ALL survival, we measured Th1, Th2, Th17 cytokines and soluble HLA-G (sHLA-G) levels in bone marrow from 32 Brazilian children at diagnosis (D0), after induction (D19) and after consolidation (D49) of the chemotherapy phase. Data were analyzed using non-parametric tests, and survival rates were evaluated by Kaplan-Meier method (log-rank test). TNF, IL-10 and IL-6 levels were increased at diagnosis compared to D19 and D49. IL-10 levels $<$ 4.57 pg/mL at diagnosis were associated with increased survival rate, in presence of positive correlation between IL-2 and IL-17 levels. Increased survival rate was also associated with IFN-γ levels $<$ 1.17 pg/mL at D49, with a positive correlation observed between IL-4 and IL-2 as well IL-4 and IL-17 levels. In contrast, worse survival rate was associated with IL-2, IL-4 and IL-10 levels imbalance. In addition, increased sHLA-G levels at diagnosis were associated with increased leukocyte count, a well-known factor for poor prognosis. In conclusion, cytokines and sHLA-G play an essential role in marrow T-ALL microenvironment during chemotherapy, especially the immunosuppressive cytokine IL-10 which can be used as biomarker of disease outcome, being also a potential target for novel T-ALL treatments.

1. Introduction

Fifteen percent of acute lymphoblastic leukemia (ALL) blasts present exacerbated proliferation and cell differentiation arrest of the T-lymphoid precursors. In spite of an increased event-free survival rate over the past decades, the T-lineage leukemia still has poorer prognosis when compared to the B-lineage ALL [1–3].

The pathogenesis of T-cell ALL involves genetic and immunological abnormalities that have been associated with environment factors such as viral infections [3–5]. Bone marrow stromal cells are the major source of cytokines and growth factors in healthy and pathological conditions. Leukemic cells can induce defective expression of soluble factors, and can change the bone marrow cytokine profile, leading to aberrant cell signaling and cell fixation, and leukemic blast proliferation in the bone marrow [6–8].

In addition to bone marrow cytokine unbalance, a differential expression of class I histocompatibility (HLA) molecules has been reported in different types of cancer [9–12]. For instance, a decreased expression of the classical HLA class I (HLA-A, B and C) molecules can decrease tumor antigen presentation to effector cells, favoring tumor development [13,14]. In parallel, an increased expression of the immune checkpoint non-classical class I HLA-G molecule has been described in several tumors [15–19]. The first evidence of the immunomodulatory properties of HLA-G was associated with the development of maternal tolerance to the fetus during pregnancy [20–23], which indicated a role of HLA-G in tumor escape from host immune surveillance [24–26].

HLA-G presents seven isoforms generated by alternative splicing.

* Corresponding author at: Laboratory of Immunogenetics/Department of Immunology, Aggeu Magalhães Research Center/Oswaldo Cruz Foundation, Avenida Professor Moraes Rego, s/n, Cidade Universitária, ZIP: 50.740-465, Recife, Pernambuco, Brazil.
E-mail addresses: norma.lucena@hotmail.com, nlucena@cpqam.fiocruz.br (N. Lucena-Silva).

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HLA-G1 to -G4 are membrane bound isoforms, and HLA-G5 to -G7 are soluble isoforms. HLA-G1 and -G5 represent the complete extracellular HLA-G molecule [24]. Soluble levels of HLA-G (sHLA-G) have been primarily detected in plasma and may vary among healthy individuals as well as in several diseases [27–30]; however, little is known about sHLA-G levels in bone marrow microenvironment. Considering that leukemic cells may propitiate a differential expression of soluble factors and considering that many cytokines may modulate sHLA-G levels, we evaluated cytokine and sHLA-G levels in the bone marrow of patients presenting childhood T-ALL at diagnosis and during chemotherapy. Since bone marrow sHLA-G levels may modulate the local immune system, we correlated soluble HLA-G and cytokine levels with T-ALL clinical features and survival rate. We observed that decreased IL-10 and IFN-γ bone marrow levels at diagnosis were associated with increased survival rate, whereas increased sHLA-G levels was associated with leukocytosis, a well-known contributor to poor prognosis.

2. Materials and methods

2.1. Patients

We studied a series of 32 children (aged 2–16 years) with T-cell acute lymphoblastic leukemia diagnosed and treated in the Pediatric Oncology Service at the Instituto de Medicina Integral Professor Fernando Figueira (IMIP) Hospital (Recife, Brazil). Bone marrow (BM) aspirates were collected at diagnosis (Day 0, n = 21), during the induction (Day 19, n = 27) and at the consolidation (Day 49, n = 24) phases. In eleven children, due to their critical clinical condition for surgical procedures at diagnosis, peripheral venous punction was performed instead of marrow punction; however, all follow-up samples were from BM. The study protocol was approved by local Oswaldo Cruz Foundation Ethics Committee and informed consent was obtained from children guardians prior to sample collection (CAAE: 13296913.3.0000.5190).

T-cell leukemia treatment protocol comprised three main steps of chemotherapy. The first step included: i) the induction phase, performed during the first 6 weeks of treatment, ii) the consolidation phase, performed during the next 8 weeks, and iii) the primary maintenance for the remaining 6 weeks. In the induction phase, children received cycles of PVDA (prednisone, vincristine, daunoblastine and asparaginase), associated to cyclophosphamide, C-aracytin and 6-mercaptopurine, and two intrathecal chemotherapy with methotrexate and C-aracytin for central nervous system protection. In the consolidation phase, patients received high doses of methotrexate and 6-mercaptopurine, while in primary maintenance, besides methotrexate and 6-mercaptopurine, patients received dexamethasone and vincristine. The second step of the chemotherapy included: (i) a re-induction-1phase for 8 weeks, when children received cycles of DVA (dexamethasone, vincristine and asparaginase), associated with daunoblastine and high doses of methotrexate and C-aracytin; and (ii) maintenance A phase for cycles of DVA associated with 6-mercaptopurine and methotrexate administration for 36 weeks. The third and last step of T-cell ALL chemotherapy included: (i) re-induction-2 phase for 4 weeks; and (ii) maintenance B with doses of 6-mercaptopurine, methotrexate, dexamethasone and vincristine for 52 weeks.

2.2. Identification and characterization of T-cell leukemia

The T-ALL diagnosis was performed evaluating BM or peripheral blood (PB) blasts that were morphologically classified according to the French-American-British (FAB) cooperative group [31]. BM and PB aliquots were submitted to cell separation by Ficoll gradient (GE Healthcare, Little Chalfont, UK) and mononuclear fraction was used for immunophenotyping analysis. T-cell lineage leukemia was confirmed by labeling mononuclear cells with fluorochrome-conjugated antibodies targeted to cell-surface and cytoplasm antigens, including the lineage specific markers CD3, CD7, CD5, CD2, CD4, CD8, CD1a, and CD99, and the immaturity markers TdT, CD34 and HLA-DR (Becton Dickinson Biosciences, San Jose, CA). Fluorescence signals were acquired using FACS Calibur flow cytometer (BD Biosciences) and analyzed with BD CellQuest™ ProSoftware (BD Biosciences). The presence of the antigen was considered to be positive when expressed in 25% or more cells. Minimal residual disease was determined by co-expression of the immature and T-specific markers identified at the diagnosis or by the presence of aberrant markers at induction (D19) and consolidation (D49) phases. The presence of at least 1% of leukemic blast was considered positive, this result was used to evaluate disease prognosis, but it did not change the therapeutic scheme.

Patient's BM cells were also submitted for molecular biology testing in order to detect the genetic errors involving the t(10;11), *HOX11*, *HOX11L2* and *SIL/TAL* alterations.

BM stroma and PB plasma were stored in aliquots at −80 °C, and were used for cytokine and sHLA-G levels evaluation.

2.3. Soluble HLA-G quantification

Soluble forms of HLA-G (shed HLA-G1 secondary to protease cleavage and HLA-G5) were quantified using a sandwich ELISA assay (BioVendor, Laboratory Medicine, Czech Republic). The experiments were performed according to the manufacturer's instructions and total sHLA-G levels were determined using a microplate reader MULTISKAN FC (Thermo Scientific, Waltham, MA) based on a six-point calibration curve, and the limit of detection was 0.6 Units/mL.

2.4. Cytokine measurements

Cytokine levels (IL-2, IL-4, IL-6, IL10, TNF, IFN-γ and IL-17A) were determined using Cytometric Bead Array Human Th1/Th2/Th17 Cytokine Kit (BD Biosciences). Quantification was performed on BD Accuri™ C6 flow cytometer (BD Biosciences), and data were analyzed using FCAP Array™ software (BD Biosciences). The assay was performed according to manufacturer's protocol. The limit of detection for each cytokine was as described in the kit protocol: IL-2 (2.6 pg/mL), IL-4 (4.9 pg/mL), IL-6 (2.4 pg/mL), IL-10 (4.5 pg/mL), TNF (3.8 pg/mL), IFN-gama (3.7 pg/mL) and IL-17 A (18.9 pg/mL).

2.5. Statistical analysis

For statistical analysis, we used the GraphPad Prism V.5.01 (GraphPad Software, Inc) and the SPSS V.20 (SAS Institute, Cary, NC) softwares. For categorical data, association analysis was performed using the Fisher's exact and the Chi-square tests. Continuous variables were analyzed using the Shapiro test, and for samples that did not follow Gaussian distribution, data were analyzed using nonparametric tests.

Differences on cytokines and sHLA-G marrow levels were evaluated by the Wilcoxon signed rank test using data from paired samples at diagnosis and day 19 of therapy (n = 17), paired samples at diagnosis and day 49 of therapy (n = 16), and paired samples from day 19 and day 49 of therapy (n = 22). Comparisons of cytokine and sHLA-G marrow levels were also evaluated in 14 samples paired for all three-time points of treatment using Friedman test and post hoc analysis to estimate differences between each two tested group combination (diagnosis × D19, diagnosis × D49, and D19 × D49).

The correlation between different cytokines and soluble HLA-G levels at diagnosis and during treatment was established by using Pearson coefficient. The linear regression analyses of the reciprocal influence of cytokine and sHLA-G levels were also performed by Pearson test; and the variables that rendered *P*-values less than 0.25 were eligible to multiple variate analysis.

For survival analyses, Kaplan-Meier method was applied with comparison of survival curves by using log-rank test. First, receiver