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SHORT PAPER

T Regulatory Cells Frequency During Maintenance Phase Chemotherapy for Pediatric Acute Lymphoblastic Leukemia

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ABSTRACT

Background: Drugs used in cancer treatment specifically kill T regulatory cells. **Objective:** To determine different phenotypes of T regulatory cells during the maintenance phase chemotherapy for pediatric acute lymphoblastic leukemia (ALL). **Materials:** We evaluated the percentages of regulatory T cells by flow cytometry. Soluble CTLA-4 (sCTLA-4) in plasma was evaluated by ELISA assay. **Results:** Increased percentages of $CD4^+CD25^+$ T cells, $CD4^+CD39^+$ T cells, $CD4^+Foxp3^+$ T cells, and $CD4^+CD25^{High}$ T cells were observed in children with ALL in comparison to healthy controls. In addition, the ALL patients with >12 months of therapy showed increased $CD4^+CD39^+$ T cells compared to the ALL patients with ≤12 months and healthy controls. Similarly, the $CD4^+CD25^+$ T cells and $CD4^+Foxp3^+$ T cells increased according to maintenance therapy time. **Conclusion:** Our results showed increased percentages of regulatory T cells in pediatric ALL patients despite chemotherapy, which might be compromising the anti-leukemic cellular immune response.

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Keywords: Acute Lymphoblastic Leukemia, ALL, Chemoterapy, Pedriatic, T Regulatory Cells

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INTRODUCTION

Regulatory T cells (Treg) are a diverse population of CD4+ T cells with anti-inflammatory properties. Treg cells may express different molecules in their membrane that confer them different immunosuppressive properties. The forkhead box transcription factor (Foxp3) is a key regulator in the development and function of regulatory T cells (1) such as anergy and dependence on IL-2 (2). CD25 is the interleukin (IL)-2 receptor α chain constitutively expressed on regulatory T cells, and the profile of CD4+ T regulatory cells can be CD25+ or $CD25^{High}$ (3). The cytotoxic T-lymphocyte antigen 4 (CTLA-4) is also constitutively expressed by regulatory T cells (4). CTLA-4 functions as a negative regulator, transmitting inhibitory signals to T cells when it interacts with B7-1/2 molecules (5). In addition, the soluble form of CTLA-4 (sCTLA-4) derived from a lack of transmembrane sequence may have important immunoregulatory functions, including interference with T cell co-stimulation and T cell responses (6). Expressed constitutively on T regulatory cells, CD39 is an ectoenzyme which converts ATP to ADP/AMP and is involved in the maintenance of low cytosolic ATP levels in these cells (7). The immune system plays a key role in cancer development because regulatory T cells suppress the activation and proliferation of conventional T cells, thereby hindering the activation of anti-tumor immune response. Diminished CD4+CD25+ regulatory T cells and increased activation of cytotoxic CD8+ T and helper CD4+ T cells with IL-2 secretion improved innate and adaptive immune responses against tumors in murine models (8). In contrast, increased number of CD4+CD25+ T regulatory with the ability to suppress CD56+ (NK) cytotoxic activity and proliferation of CD4+ T cells in patients with epithelial malignancies prevented anti-tumor responses (9). Moreover, higher levels of CD4+CD25+ and T-regulatory-1 (Tr1) cells induced an immunosuppressive environment in Hodgkin lymphoma patients, which was ameliorated by neutralizing IL-10 and blocking CTLA-4 (10). Furthermore, previous studies demonstrated a change in the regulatory T cells in leukemia patients. In this sense, higher proportions of $CD4+CD25^{high}$Foxp3+ cells and Foxp3 expression were observed in pediatric B-cell acute lymphoblastic leukemia (11). And, individuals with chronic lymphoblastic leukemia (CLL) showed an increase in CD4+CD39+ lymphocytes which correlated with the advanced disease stage and poor prognosis (12). Similarly, contributing to anti-tumor response impairment, a low percentage of regulatory T cells with CD62L co-expression of were observed in children with acute lymphoblastic leukemia (ALL). CD62L is shed from the cell membrane following T cell activation, meaning most of the regulatory T cells were activated in ALL patients, which might be an immunosuppression mechanism accompanying acute lymphoblastic leukemia (13). Taken together, this data supports the notion that Tregs lead to impaired anti-leukemic cellular immune response. In a previous study, the levels of CD4+CD25+Foxp3+ cells decreased following chemotherapy in patients with acute leukemia, suggesting that their detection might be considered to monitor the immune status of patients (14). Additionally, cyclophasphamide, a drug used in the treatment of cancer, selectively killed T regulatory cells since they showed reduced intracellular ATP levels (7). Confirming this result, reports have proven that the administration of cyclophosphamide depletes CD4+CD25+ T cells and allows for tumor rejection (15). Considering this data, in the present study, we evaluated the percentage of different T regulatory cells, including CD4+CD39+ T cells, CD4+CD25+ T cells, $CD4+CD25^{high}$ T cells,

CD4+Foxp3+ T cells, CD4+CTLA-4+ T cells and plasma levels of soluble CTLA-4 in children undergoing chemotherapy for acute lymphoblastic leukemia in the maintenance phase.

MATERIALS AND METHODS

Individuals. ALL patients (n=14 with 7.8 ± 4.2 years) were recruited from Hospital General Zacatecas, Zacatecas, Zac, Mexico. An ALL diagnosis was obtained from morphologic analysis of bone marrow (BM) aspirates. Only six patients were categorized for different classes of ALL. Three patients were considered as mature B-cell ALL, two were classified as pre T-cell ALL, and one was diagnosed with Precursor B-cell ALL. ALL cases were in the maintenance phase of the treatment, comprising Citarabina, Etoposide, Cyclophosphamide, Vincristine, Methotrexate, and Hydrocortisone. Furthermore, the ALL cases were classified according to their chemotherapy time course; therefore, children with ≤12 months of treatment were allocated into one group and children with >12 months of therapy were allocated into another. A healthy control group consisting of 13 children (13.6 ± 4.05 years) was included. General characteristics of each group are described in Table1. The Bioethics Committee of the Zacatecas University approved the study protocol (project number UAZ-2013-362664), and written informed consent was obtained from all legal tutors of the participants.

Table 1. Main characteristics of acute lymphoblastic leukemia patients and healthy controls.

	ALL Patients	Control Subjects
N	14	13
Sex (F/M)	6/8	8/5
Age (Years)	7.8 ± 4.2	13.6 ± 4.05*
Evolution (Months)	23.6 ± 13.3	-
Treatment	Maintenance+	None

+Maintenance chemotherapy consists: Citarabina, Etoposide, Cyclophosphamide, Vincristine, Methotrexate and Hydrocortisone. ALL: acute lymphoblastic leukemia, F: Female, M: Male. Data correspond to the arithmetic mean±SD. Significant differences were detected using the un-paired t test. * $p<0.05$

Peripheral Blood Mononuclear Cell Isolation. Blood samples from ALL patients and control subjects were centrifuged for 10 minutes at 1500 rpm to obtain plasma. Afterwards, the cellular package was diluted in 4 mL of phosphate buffered saline (PBS) and placed over Ficoll–Paque gradient (GE Healthcare Bio-Sciences AB, Sweden). The peripheral blood mononuclear cells (PBMC) layer was isolated and washed with PBS. The cells were adjusted at 1×10^6 cells/mL in PBS. The viability of PBMC was assessed by trypan blue exclusion assay. A viability above 99% was considered acceptable.

Flow Cytometry Analysis. Cells (5×10^5 cells/μL) were immunostained with 0.5 μg/mL anti-CD4 conjugated with fluorescein isothiocyanate (FITC) and anti-CD39 conjugated with allophycocyanin (APC) or anti-CD25 conjugated with APC for 30

minutes; they were then washed with PBS and fixed with 1% p-formaldehyde (PFA). For the detection of intracellular antigens, use was made of specific mAbs for CTLA-4 (BD PharMingen, BD Biosciences San Jose, CA, USA) or Foxp3 (PCH10 clone; eBioscience, San Diego, CA, USA). A double-labeling procedure was performed with anti-CD4-FITC, followed by fixation and permeabilization with 4% PFA and 0.01% saponin or fixation-permeabilization buffers supplied by eBioscience. Finally, the cells were stained with anti-CTLA-4-PE or anti-Foxp3-PE mAbs. They were analyzed in FACSCanto II flow cytometer using the Diva software (BD Biosciences, San Jose, CA, USA)

Soluble CTLA-Level Determination. Soluble CTLA-4 (sCTLA-4) was determined in the plasma of ALL patients and healthy controls using the ELISA assay (Human sCTLA-4 Platinum ELISA, BMS276, eBioscience, Vienna, Austria). Assay sensitivity was 0.13 ng/mL and carried out according to the manufacturer's protocol; the signal was further measured on a microplate photometer (Multiskan® FC, Thermo Scientific, USA).

Statistical Analysis. Statistical analysis was performed using GraphPad Prism 5.0 software (San Diego, CA, USA) To evaluate the differences between the means, Mann Whitney U test and Kruskal-Wallis test were performed. $P<0.05$ was considered as significant.

RESULTS and DISCUSSION

Percentages of T regulatory cells in children with maintenance phase chemotherapy for pediatric ALL and healthy controls.

Figure 1 shows the flow cytometry (FC) analysis and gating strategy of the identified CD4+CD25+ T regulatory cells (PBMC). We detected increased percentages of CD4+CD25+T cells (Figure 1b), CD4+CD39+ T cells (Figure 1c), CD4+Foxp3+ T cells (Figure 1d), and CD4+CD25^{high} T cells (Figure 1e) in ALL children compared with healthy controls. We further observed an increase in CD4+CD25+ T cells (Figure 2a), CD4+CD39+ T (Figure 2b) cells, and CD4+Foxp3+ cells (Figure 2c) of ALL patients with >12 months of maintenance chemotherapy compared to ALL patients with ≤ 12 months (Figure 3c). According to our results, a higher number of myeloid-derived suppressor cells (MDSC) were observed during and after the induction of chemotherapy for B-ALL (16). Moreover, previous studies showed that cyclophosphamide failed to diminish T regulatory numbers and function in patients with metastatic carcinoma (17). Moreover, increased CD4+CD39+ cells were seen in CLL patients with poor prognosis and in need of therapy (12). CD39 and CD73 generated adenosine from ATP, adenosine suppress T cells, and Natural Killer (NK) cells anti-tumor activity (18). On the contrary, we observed similar percentages of CD4+CTLA-4+ T cells (Figure 1f) and plasma levels of soluble CTLA-4 (sCTLA-4) (Figure 1f) in ALL cases and healthy controls. In addition, augmented levels of sCTLA-4 were found in ALL patients with >12 months of chemotherapy compared to ALL patients with ≤ 12 months of treatment and healthy controls (Figure 1f). Previous studies reported that most ALL pediatric patients with active disease had elevated levels of serum sCTLA-4, correlated with CD1d expression and, consequently, poor prognosis (19,20).

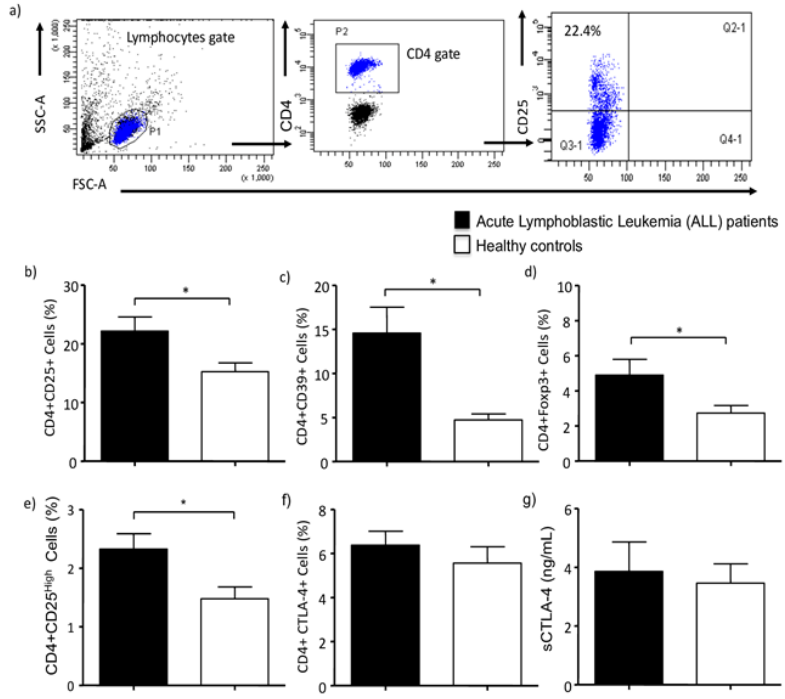

Figure 1. Flow cytometry (FC) analysis of peripheral blood monocyte cells (PBMC). The percentage of T regulatory cells was determined through analyzing the percentage of CD25, CD25^{High}, Foxp3, CTLA-4, and CD39 gated on CD4+ T cells. Representative dot-plots of CD4+ T cells expressing CD25 of one ALL patient is shown in (a). The percentages of T regulatory cells are shown in b) CD4+CD25+ T cells, c) CD4+CD39+ T cells, d) CD4+Foxp3+ T cells, e) CD4+CD25High T cells, and f) CD4+CTLA-4+ T cells. Levels of soluble CTLA-4 (sCTLA-4) in plasma are shown in g). Data correspond to mean ± SEM. Statistical analysis was performed with Mann Whitney U test. *; p<0.05.

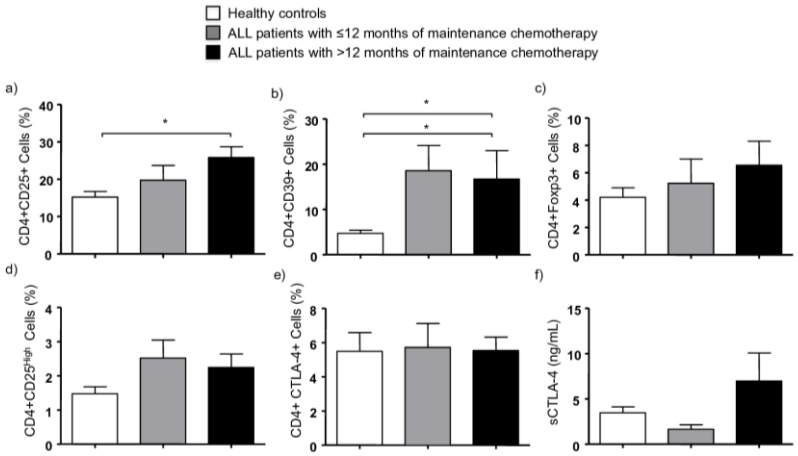

Figure 2. The percentages of T regulatory cells belonging to ALL patients classified according to their chemotherapy time course. The ALL cases were classified as children with ≤12 months of treatment (n=4) and children with >12 months of therapy (n=10). a) CD4+CD25+ T cells, b) CD4+CD39+ T cells, c) CD4+Foxp3+ T cells, d) CD4+CD25High T cells, and e) CD4+CTLA-4+ T cells. Levels of soluble CTLA-4 (sCTLA-4) in plasma are shown in f). Data correspond to mean ± SEM. *; p<0.05.

Based on these results, despite chemotherapy, increased T regulatory cells co-expressing CD39, CD25 and Foxp3, and sCTLA-4 are present in children with ALL. Finally, we propose that new treatment strategies such as therapy combined with drugs-specific against T regulatory-cells could be more effective.

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