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Evaluation of interleukin 12 and CD56+ lymphocyte cells in pediatric hematopoietic stem cell transplantation for early diagnosis of acute graft versus host disease

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ABSTRACT

The present study tried to explain CD56+ lymphocyte cells activities and possible prognostic role of these cells in Graft-Versus-Host-Disease (GVHD). The role of IL-12 activation and function is of interest in this study. Peripheral blood samples of 51 Hematopoietic Stem Cell Transplantation (HSCT) recipients collected at before (day −8) and after (days 7 and 14). PBMC were collected by Ficoll separation and analyzed by Flow Cytometry using triple antibody (CD45-PerCP, CD56-FITC, and CD69-PE staining and control antibody. Levels of the cytokine IL-12 in the patient's serum were evaluated by ELISA. Percentage of CD56+ lymphocytes (CD56+^{bright}) cells was significantly increased at day 14 in patients with acute GVHD and percentage of lymphocytes expressing CD69 was significantly increased at days 7 and 14 posts HSCT in patients with acute GVHD in comparison to those in non-GVHD patients. Baseline serum IL-12 levels (pre-HSCT, day −8) were significantly higher in those HSCT recipients who did not develop GVHD. This study showed that post-transplant CD56+ lymphocytes and pre-transplant serum levels of IL-12 play significant roles in the induction of and protection against GVHD, respectively. The increase in the percentage of CD69+ cells indicates the activation of lymphocyte in acute GVHD group.
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1. Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is currently applied as a curative treatment for a wide range of diseases including hematological malignancies (e.g., leukemia and lymphomas), solid tumors and non-malignant diseases such as severe aplastic anemia, autoimmune diseases, congenital immunodeficiency syndromes and metabolic disorders in children [1,2]. As a desirable effect, allogeneic HSCT can promote graft versus leukemia (GVL) which is mediated by donor T cells [3]. However, allo-reactive donor T cells can also induce acute and chronic graft-versus-host disease (GVHD) [4–6]. In fact, GVHD represents a major complication of allogeneic HSCT, which results in significant morbidity and mortality, thereby limiting the use of this treatment procedure as a curative approach.

Studies have shown that GVHD develops as a three consecutive phases [6–8]. The first phase involves damage to host tissues and induction of cytokine storm as a result of pre-transplant conditioning. In the second phase, both recipient and donor antigen presenting cells, and inflammatory cytokines trigger activation of donor-derived T-cells that expand and differentiate into effector cells. In this activation phase, minor histocompatibility antigens play a central role, particularly in the setting of matched sibling transplants. T-cell activation pathways results in the transcription of genes for inflammatory cytokines, such as, interleukin-2 (IL-2) and interferon-gamma (IFN-g). T-cells that produce these cytokines are considered to be Th1 phenotype [6–8]. Finally, in the third phase, In the third (effector) phase, activated donor T-cells mediate cytotoxicity against target host cells through Fas–Fas ligand interactions, perforin–granzyme B and the additional production of cytokines, such as, tumor necrosis factor alpha(TNFα) [9]. Intestine, skin, liver, and lungs are the most frequently affected organs, which are assaulted by alloreactive donor T-cells [10].

The development and severity of GVHD depend on several factors including; the intensity of pre-transplant conditioning regimen, presence and the number of donor T cells in the graft, and antigenic disparity between donor and recipient [7,11–13]. Furthermore, GVHD can occur in any type of allogeneic setting regardless of conditioning protocol or

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even in the absence of conditioning [5,14–17]. In addition to donor T cells, the innate immune related lymphocytes such as natural killer (NK) cells may also influence the outcome of GVHD [18]. For instance, animal studies have shown that NK cells are able to facilitate engraftment and augment graft-versus-tumor effects [9,19,20]. In this connection, results of several clinical studies have revealed that an increase in NK-activating receptor activity or a reduction in signaling of inhibitory NK receptors improves the outcome of HSCT [21–23]. Furthermore, interleukin 12 (IL-12), a cytokine produced by monocytes-macrophages, dendritic cells, and other antigen-presenting cells [24] has been shown to induce a protective effect on the development of acute GVHD in a murine model of this disease [25].

In the light of these effects of NK cells and IL-12 on the outcome of HSCT in experimental models, we asked whether these factors also play a role in the development of HSCT-related complications in pediatric patients. Therefore, the present study was undertaken to assess the relationship between the serum IL-12 concentrations, NK cells and occurrence of GVHD in children receiving HSCT.

2. Materials and methods

2.1. Patients

Fifty one children with different hematological malignancies and diseases were enrolled in the present investigation. The patients were admitted at Cancer Research Center, Tehran University of Medical Sciences. Twenty four of these patients were diagnosed with thalassemia, five with Fanconi anemia, seven with acute myelogenous leukemia (AML), four with acute lymphoblastic leukemia (ALL), three with aplastic anemia, three with chronic granulomatous disease (CGD), one with sickle cell anemia, one with diamond–Blackfan anemia (DBA), one with myelodysplastic syndromes (MDS), one with Chronic Myelomonocytic Leukemia (CMML) and one with Leukocyte adhesion deficiency-1 (LAD-1). Among these patients, 14 patients developed acute GVHD after HSCT and the rest remained disease free until day 100 after HSCT. Acute GVHD was diagnosed on the basis of clinical symptoms and/or biopsies (skin, liver, gastrointestinal tract, or oral mucosa) according to standard criteria [26]. Patients' characteristics are presented in Table 1.

2.2. Blood sampling

At different time points, i.e., before (day −8) and after (days 7 and 14) HSCT, Two tubes at 2,5 ml blood were sampled from each patient, which equaled 5 ml per person. Tube one contained ethylene diamine tetra-acetic acid (EDTA) as anticoagulant, whereas tube two was without EDTA. From tube one, peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats using Ficoll density gradient separation in accordance with the manufacturer's instruction (Lymphodex, Inno-Train, REF:002041600). Serum samples from tube two were separated after centrifugation at 1500 rpm for 10 min, and then it was stored at −80 °C till further analysis.

Table 1
Patient and transplant characteristics.

Male	35
Female	16
Disease	
Thalassemia	24
Fanconi anemia	5
AML	7
ALL	4
Other (LAD = 1, CMML = 1, CGD = 3, Aplastic Anemia = 3, Sickle cell anemia = 1, DBA = 1, MDS = 1)	11
Type of graft	
Allo B.M.[a]	
Allo P.B.[b]	
Cord blood	
Acute GVHD	14
Non-GVHD	37

[a] Allo B. M.: allogenic bone marrow.
[b] Allo P.B.: allogenic peripheral blood.

2.3. Detection of serum interleukin IL-12 (p70)

Stored serum samples at −80 °C were used to determine the concentrations of IL-12(p70) using enzyme-linked immunosorbent assay (ELISA) in accordance with the manufacturer's instructions (Mabtech AB, Nacka Strand, Sweden).

2.4. Immunofluorescent staining and flow cytometric analysis

For immunophenotyping of CD56+ lymphocytes and activated these cells, one million PBMC cells were incubated with anti-human CD56-FITC, anti-human CD69-PE and anti-humanCD45-PerCPin accordance with the recommendations of the manufacturer (BD, San Diego, CA). The erythrocytes were lysed employing the Uti-Lyse Erythrocyte lysing reagent in accordance with the manufacturer's instruction (Dako Denmark A/S). The data were analyzed using a Partec Flow Cytometry device and the Flow Max software.

2.5. Statistical analysis

All data are expressed as Mean ± SEM (standard error) unless otherwise mentioned. Differences between allogeneic and syngeneic analyzed using Mann–Whitney (*U* test).

$P < 0.05$ is considered statistically significant. All statistical analyses were performed utilizing SPSS ver13.

3. Results

3.1. The role of CD56+ lymphocyte in the development of acute GVHD

CD56+ lymphocytes and activated (CD56+CD69+) frequency were determined at days, −8, 7 and 14 prior and after allogeneic HSCT, respectively (Fig. 1a-l). Blood leukocytes were first identified by gating on CD45 positive cells (Fig. 1e, h and k) and thereafter, lymphocytes expressing CD56 and CD69 were identified on the gated CD45+ cells (Fig. 1f, I and l). Our results showed that the percentage of CD56+ lymphocytes was significantly increased 7 days after HSCT in both non-GVHD and GVHD groups as compared to day −8 prior to HSCT (Fig. 2a). However, at day 14 post HSCT, the frequency of CD56+ cells in patients with acute GVHD was markedly higher than those in non-GVHD patients (Fig. 2a). On the other hand, we observed that the frequency of CD56+ lymphocytes expressing CD69 in patients with acute GVHD was similar to those in non-GVHD patients at all tested time points (Fig. 2b). However, the percentage of total cells expressing CD69 was significantly increased at days 7 and 14 post HSCT in patients with acute GVHD in comparison to those in non-GVHD patients (Fig. 2c).

3.2. The role of baseline (pre-HSCT) IL-12 levels in the development of acute GVHD

Next, we analyzed whether the baseline levels of serum IL-12 levels would affect the outcome of HSCT in connection with the development of GVHD. As shown in Fig. 3, the baseline serum IL-12 levels (pre-HSCT, day −8) were significantly higher in those HSCT recipients who did not develop GVHD as compared to those in patients with acute GVHD.

4. Discussion

Acute GVHD is a complex inflammatory process in which several factors including the immune status of the recipient, pre-transplant

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