Case Report

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Outcome of Cyclophosphamide Treatment Following Hematopoietic Stem Cell Transplantation in a Thalassemia Patient: A Case Study

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ABSTRACT

Hematopoietic stem cell transplantation (HSCT) is the only curative therapy for β-thalassemia major in children. However, it often induces graft-versus-host-disease (GVHD), which is associated with complications. In the present study, we used cyclophosphamide (Cy) to treat a thalassemia patient post-HSCT to reduce the adverse effects of GVHD. We monitored the numbers and phenotype of granulocytes. In this case study, an 11-year-old female patient, diagnosed with β-thalassemia major (Pesaro class II), was treated with Cy before and after HSCT with mobilized $CD34^+$ cells. Both the relative and absolute granulocyte counts, as well as $CD33^+CD11b^+$ cell counts, increased significantly after HSCT until day 56. However, they suddenly began to decrease after day 56, accompanied by severe diarrhea, skin rash, and a decrease in bilirubin levels compared to day -12. Furthermore, compared to day -12, IL-22 levels increased until day 56, and then decreased, while IDO levels continued to rise after day 56. Our data suggest the potential use of IL-22 and IDO as biomarkers for GVHD assessment. It also indicates that Cy promotes HSCT reconstitution by increasing $CD33^+CD11b^+$ cells, which may play a crucial role in reducing GVHD risks. However, further studies are needed to elucidate the mechanism behind GVHD recurrence.

Keywords: B-thalassemia, Granulocytes, Cyclophosphamide, GVHD, HSCT

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INTRODUCTION

The only curative treatment for β-thalassemia major is hematopoietic stem cell transplantation (HSCT), chosen over iron chelation and blood transfusions to prolong life in these patients (1). The most well-known complication following HSCT is the graft versus host disease (GVHD) (2). GVHD in thalassemia patients can affect the heart and a variety of other body organs, resulting in death, along with relapse, infections, and hemorrhagic cystitis (3).

In a series of preclinical and clinical studies, including ours, cyclophosphamide (Cy), a conventional anticancer drug, has

been used as a beneficial preconditioning regimen before adoptive T cell therapy (4). Cy is also a well-established agent that provides immunosuppression for allogeneic engraftment (5). We and others have found that post-Cy treatment is associated with significant expansion of a subpopulation of granulocytes called myeloid-derived suppressor cells (MDSCs) during the recovery from leukopenia (4, 6, 7).

MDSCs are a diverse population of naturally occurring immature myeloid cells characterized by their capacity to suppress responses mediated by both innate and adaptive immunity. MDSCs arise under chronic inflammatory conditions such as malignancy, infection, autoimmune diseases, trauma, and GVHD. Although they are phenotypically like mature myeloid cells, they pose distinct genomic and biochemical profiles and function (7). MDSCs can be broadly categorized into two groups: Monocytic (M-MDSC) and polymorphonuclear (PMN-MDSC). The latter are also known as granulocytic MDSCs (G-MDSCs) (8). Both subtypes of MDSC are phenotypically and morphologically analogous to neutrophils and monocytes, respectively (9).

Granulocytes are a diverse population of innate immune cells that expand during malignancies and have a remarkable capacity to suppress T cell responses (10). Our group reported high numbers of granulocytes with MDSC phenotype in the peripheral blood of breast cancer patients (11), hepatitis C virus patients (12), cirrhosis and hepatocellular carcinoma patients (13), and acute lymphoblastic B-cell leukemia patients (14). Also, other studies reported high numbers of these cells in the colon, pancreatic, and premalignant intraductal papillary mucinous neoplasms cancer patients (15). In cancer, most MDSCs are represented by cells with granulocytic phenotype and morphology, G-MDSC (16).

The immunosuppressive capacity of CD33+CD11b+ cell is induced upon secretion of immunoregulatory mediators such as indoleamine 2,3-dioxygenase (IDO) as well as interlukin-22 (IL-22) (17). IDO is an intracellular heme-containing enzyme that catalyzes the first and rate limiting step of tryptophan catabolism along the kynurenine pathway (18). Increased IDO activity is related to many diseases, such as inflammatory diseases, cancer, liver diseases, diabetes, depression, HIV and rejection of organ transplants (1). IDO also leads to the depletion of the essential amino acid tryptophan that induces $CD33^{+}CD11b^{+}$ recruitment and the suppression of immune responses (19). IL-22 is a recently described IL-10 family cytokine produced by T-helper (Th)-17 cells, γδ T cells, NKT cells and the innate lymphoid cells (ILCs). IL-22 has also been linked to several conditions involving inflammatory tissue pathology (20), and chronic inflammatory diseases, including rheumatoid arthritis and inflammatory bowel disease, resulting in the downregulation of the associated immune responses (21). As such, these two mediators, IL-22 and IDO, may play a role in the modulation of GVHD.

The standard protocol to reduce GVHD is the use of anti-thymocyte globulin (ATG) (22). However, recent studies in several centers have been using Cy post GVHD (PTCy) instead of ATG, which associate with a lower incidence of GVHD, providing a better long-term disease control (23).

Given the importance of granulocytes, IL-22, and IDO in immunoregulation, we thought to analyze the changes in the numbers and phenotype of granulocytes as well as the levels of IL-22 and IDO in a patient with thalassemia throughout the 84 days of follow-up even in recurrence of GVHD after Cy treatment in correlation with the clinical data.

THE PATIENT AND METHODS

An 11-year-old female patient diagnosed with β-thalassemia (Pesaro class II) by gene testing for Hb, was admitted to Bone Marrow

Transplantation Unit, Tanta University Teaching Hospital, Egypt. Cy was given to the patient for 4 consecutive days before the HSC transplantation (i.e. on days 5, 4, 3 and 2), and then 2 days post HSC transplantation (i.e. on days 3 and 4). On day 0, the patient received $CD34^+$ mobilized blood (4.7 million cell/Kg) from the donor, her 7-year-old older sister treated previously with granulocyte colony stimulating factor (G-CSF) (Neupegen®) subcutaneously once a day for 5 consecutive days. The blood samples were collected from the patient on day -12 (before transplantation), 0 (the transplantation day), 12 (one week after the last PTCy dose), 19 and 26 (with one-week intervals in between). After ending the typical course of treatment around day 56, the patient suffered from GVHD based on the clinical diagnosis as she suffered from severe diarrhea that did not respond to any antibiotics, as well as severe skin rash that did not respond to antihistaminic drugs, and severe decrease in the bilirubin level. The prophylactic antibiotic and anti-fungal used was ciprofloxacin and dilflucan. It is likely that the cause of the death is the recurrent viral (73.3%) and bacterial infection (53.3%). The blood samples were collected on days 63, 70, 77 and 84 (with one-week intervals in between) as shown in Fig. 1. Flow cytometry was used to analyze the phenotype of granulocytes after staining the cells with mAbs against CD33 and CD11b. The total WBCs were counted in the peripheral blood using an automated instrument to complete the blood count (CBC). The plasma was obtained from the patient through centrifugation and stored at 80 °C until tested. IDO and IL-22 were measured by ELISA.

THE RESULTS AND DISCUSSION

The assessment of the hematological indices throughout the follow-up days showed improvement in the levels of hemoglobin (Hb), decreases in the numbers of platelets with no changes in the numbers of RBCs compared with day -12 (as the base level) shown in Table 1. The total number of leukocytes, and both the relative and absolute numbers of lymphocytes decreased during the follow-up days compared with day -12. While the relative and absolute numbers of granulocyte significantly increased during the follow-up days PTCy compared with day -12, only the absolute, but not the relative, numbers of monocytes decreased, shown in Table 2.

Granulocytes in the patient were identified by flow cytometry as $CD11b^+CD33^+$ cells. These cells were gated from total forward and side scattering (TLC) or from either

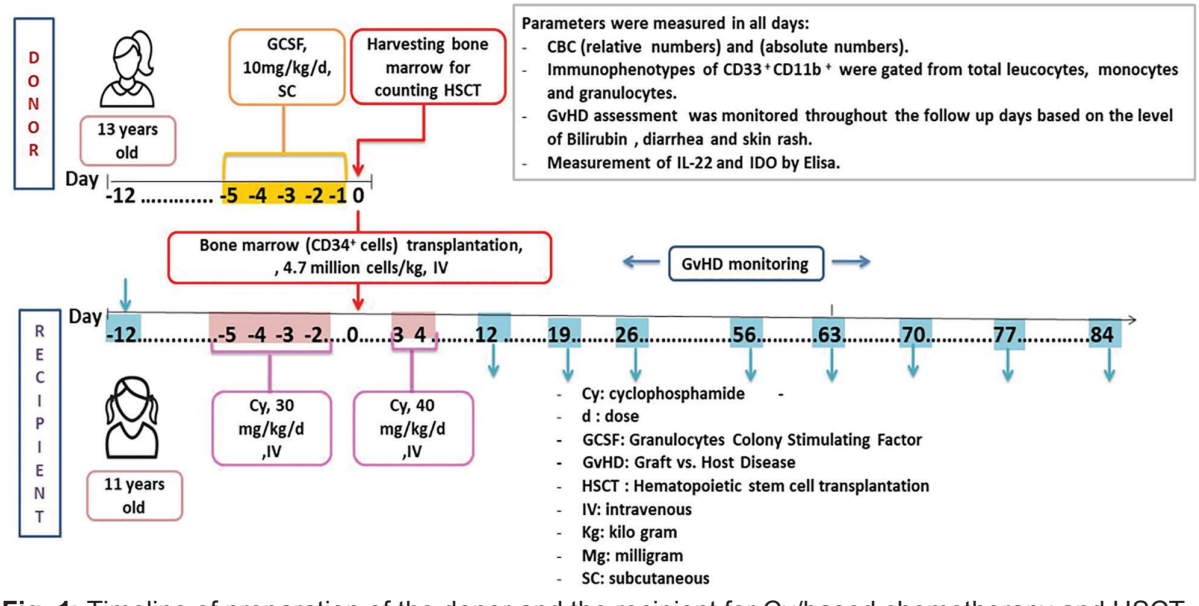

Fig. 1: Timeline of preparation of the donor and the recipient for Cy/based chemotherapy and HSCT.

Table 1: Hematological indices in the patient

Days of analysis	RBC (10^6/cmm)	Hg (g/dl)	PLT (10^3/cmm)
Day -12	3.5	7.9	316
Day 0	2.8	6.8	133
Day -12	4.5	12.6	132
Day 19	3.9	10.6	30
Day 26	3.1	7.9	98
Day 56	3.2	8.2	50
Day 63	3.4	8.6	50
Day 70	2.7	7.2	60
Day 77	3.7	9	70
Day 84	3.3	8.7	110

Table 2: Total number of leukocytes and the relative and absolute numbers of lymphocytes, monocytes, and granulocytes in the patient

CBC count	Total WBCs 10^3/mm3	Absolute Lymphocytes 103/uL	Relative Lymphocytes 103/uL	Absolute Monocytes 10^3/uL	Relative Monocytes 103/uL	Absolute Granulocytes 10^3/uL	Relative Granulocytes 10^3/uL
Day -12	5.3	3.18	60	0.21	4	0.91	36
Day 0	2.5	0.5	20	0.1	4	1.9	76
Day -12	1.2	0.1	8	0.06	5	1.04	87
Day 19	1.9	0.19	10	0.1	5	1.62	85
Day 26	2	0.24	12	0.1	5	1.66	83
Day 56	3.3	0.33	10	0.16	5	2.8	85
Day 63	1.9	0.19	10	0.1	5	1.62	85
Day 70	2.2	0.22	10	0.11	5	1.87	85
Day 77	3.1	0.93	30	0.12	4	2.05	66
Day 84	2.3	0.6	26	0.09	4	1.56	68

granulocyte or monocyte populations. This gating strategy is shown in Fig. 2. We found that the relative and absolute numbers of $CD33^+CD11b^+$ gated from TLC, as well as granulocytic and monocytic populations showed increases until day 56 then it started to decrease (Figs. 3A and B). In the follow-up days until day 26 (a good prognosis), there was an inverse correlation between CRP (as inflammatory marker) and $CD33^+CD11b^+$. While, after day 26 (a bad prognosis) there was a direct correlation between CRP and $CD33^+CD11b^+$ (data not shown). The level of IL-22 showed increases until day 56 then it started to decrease until day 84 (Fig. 4A), while the level of IDO increased only after day 56 (Fig. 4B).

We report in the present study that the relative numbers of granulocytes based on CBC analysis significantly increased after HSC transplantation and PTCy. Similarly, the relative numbers of $CD33^+CD11b^+$ cells gated from TLC, monocytic or granulocytic populations also increased until day 56 then it started to decrease. In the context of allo-HSCT, we suggest that $CD33^+CD11b^+$ cells, both in the donor graft and in the recipient post allo-HSCT, could be beneficial in establishing the graft by mediating immune regulation. A recent study has found that $CD33^+CD11b^+$ cells promote transplant tolerance and may therefore help control GVHD (24). Prior reports have demonstrated that $CD33^+CD11b^+$ cells are one of the earliest donor-derived cell types to recover after allo-HSCT (25). These cells have been found to alleviate GVHD after allogeneic bone marrow transplantation (18). In this context, our group reported the presence of high numbers of $CD33^+CD11b^+$ cells in the peripheral blood of patients with

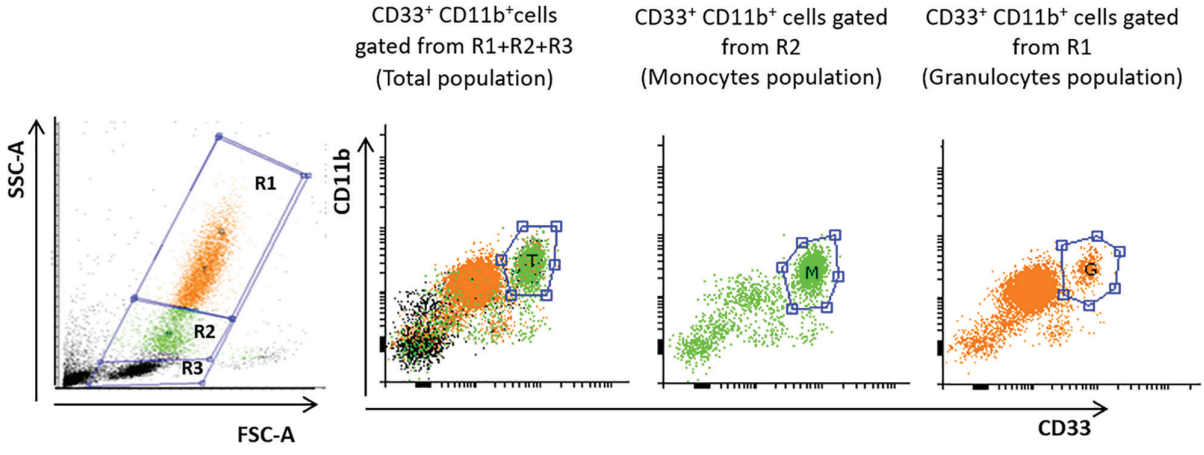

Fig. 2: Gating strategy of CD33⁺CD11b⁺ cells in the peripheral blood using flow cytometry gated from total population (TLC), monocytes (M), and granulocytes (G).

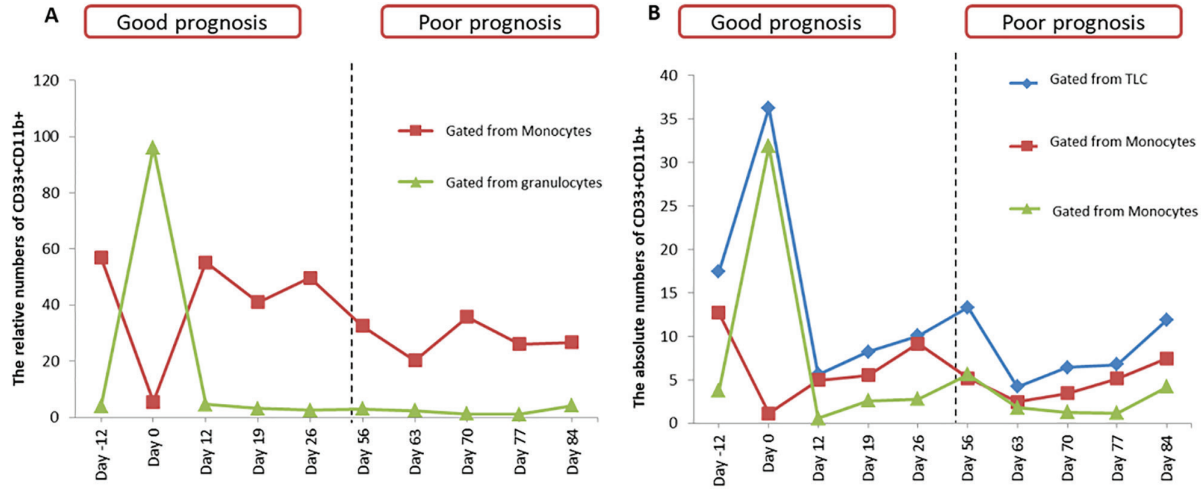

Fig. 3: The relative and absolute numbers of CD33⁺CD11b⁺ cells in the peripheral blood. (A) The relative numbers gated from monocytes and granulocytes populations (B) The absolute numbers gated from TLC, monocytes, and granulocytes in PTCY recipient. The blood samples were collected from the patients on day -12 (before transplantation), day 0 (the transplantation day), day -12 (one week after the last Cy dose), days 19, 26, 56, 63, 70, 77 and 84 (with one-week intervals in between), Cy 30 mg/Kg/dose was given to the recipient for 4 days before the transplantation (on days 5, 4, 3 and 2), and PTCy was given to the donor for 2 days after the transplantation 40 mg/Kg/dose (on days 3 and 4). The cells were stained with anti-CD33 and anti-CD11b mAbs and then acquired by flow cytometry and analyzed by FlowJow software.

cirrhosis and hepatocellular carcinoma (13). Similar results were found in hepatitis C virus (12), and B-cell acute lymphoblastic leukemia patients (14). As such, it is expected in the context of allo-HSCT that granulocytes are beneficial to the host by inhibiting the grafted T cells to attack the host as well as maintaining the survival of the donor cells.

Our results regarding IL-22 showed that its levels started to decrease after day 56 when the patient started to suffer from GVHD, indicating that high levels of IL-22 during acute GVHD are beneficial to lower the associated adverse effects. In line with our hypothesis, it has been reported that the levels of IL-22 in GVHD patients suffering from transplant-related mortality (TRM) were significantly lower than in patients with less TRM (26). Taken together, it can be suggested that IL-22 plays an essential role of IL-22 in the prognosis of patients undergoing allo-HSCT. In contrast, we found increases in the IDO levels after day 56 the time point when the patient started to suffer

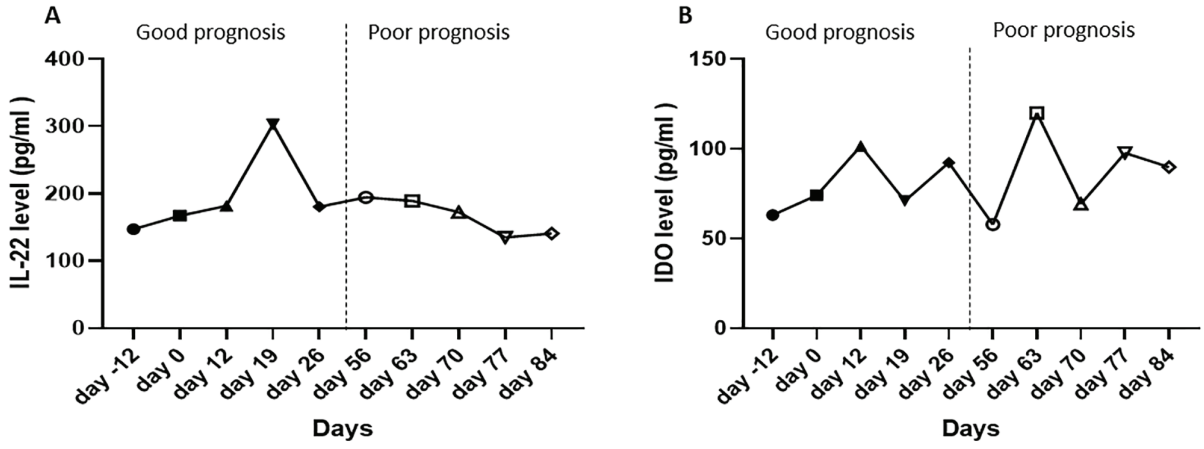

Fig. 4: Plasma Levels of IL-22 and IDO in the patient. Plasma samples were prepared from the patient at the same time and the treatment conditions indicated in Fig. 3. Then, the levels of IL-22 and the activity of IDO were measured by ELISA kits.

from GVHD. Consistent with our results of IDO, a previous study showed that the plasma levels of IDO activity were elevated in acute-GVHD (a-GVHD) patients and correlated with the severity of aGVHD (18).

Taken together, our findings indicate the important role of granulocytic $CD33^{+}CD11b^{+}$ population after post Cy treatment in mediating protection from GVHD following allo-HSCT. Exploring strategies that can enhance the role of these cells during the hematopoietic recovery might provide further insight into the immunoregulation of PTCy microenvironment. It can also be suggested that both IDO and IL-22 might represent potential biomarkers for the diagnosis and evaluation of aGVHD after allo-HSCT. Yet, further studies are needed to confirm our hypotheses and GVHD post Cy presented in this case study.

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AUTHORS' CONTRIBUTION

HMB was responsible for conducting the experiment and compiling the raw data. SMK designed the study, participated in the execution of the experiments, performed data analysis and interpretation, and drafted the initial manuscript. MRE supplied the blood samples from donor and patient, monitored the clinical presentations of the patient, and assisted in revising the manuscript. MLS provided the technical resources for the experiments, originated the concept, formulated the experimental design, and reviewed the manuscript. All authors approved the data in the manuscript for publication.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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