REAL-LIFE DATA ON IMMUNE RECONSTITUTION AFTER ALLOGENIC STEM CELL TRANSPLANTATION: AN OBSERVATIONAL STUDY IN PEDIATRIC PATIENTS

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Abstract

Background: Immune reconstitution (IR) after allogenic hematopoietic stem cell transplantation (allo-HSCT) is a long and progressive process intrinsically correlated to therapeutic success. It is essential to understand interfering factors in IR to prevent HSCT-related mortality. Methods: We retrospectively evaluated absolute lymphocyte count (ALC) and lymphocyte subtypes of 111 pediatric patients with allogeneic HSCT for malignant and non-malignant diseases from 2013 to 2018. ALC recovery on day +30 (D+30), +100 (D+100) and +180 (D+180) and subtypes CD3+CD4+, CD3+CD8+, CD19+ and CD16+CD56+ on D+100 were correlated to the HSCT procedure, clinical outcomes, and survival. Results: ALC had a gradual increase on D+30, D+100 and D+180 (medians 634/µL, 1 022/µL and 1 541/µL, respectively). On D+100, CD3+CD8+ achieved the highest recovery rate (68%), followed by CD16+CD56+ (47%), CD3+CD4+ (39%) and CD19+ (8%). Adequate ALC recovery on D+30 was associated with age <8 years, bone marrow grafts, myeloablative conditioning, and non-haploidentical donors. The use of serotherapy correlated to a poor ALC recovery on D+180. Counts of ALC and CD3+CD8+ on D+100 were higher in patients with cytomegalovirus infection. CD3+CD4+ recovery was associated with age <8 years, non-malignant disease and a lower incidence of acute graft-versus-host disease [?]grade 2. Further, ALC and CD3+CD4+ recovery on D+100 resulted in higher overall survival, as ALC was determinant regardless of disease type (HR 3.65, 1.05-12.71, P=0.04). Conclusion: Several factors influenced IR after allo-HSCT. ALC[?]500/µL on D+100 was found to be a simple IR biomarker and a good predictor of survival, easily available to resource-limited countries.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a potential treatment for many patients with malignant and non-malignant diseases. Although much improvement has been made during the past decades, several complications affecting patient survival or quality of life remain to be addressed.[1]

It is well established that immune reconstitution (IR) after HSCT is influenced by patient and transplant factors and strongly associated with transplant outcomes. [2] Disease type, recipient age, source of the stem cells, type of donor, conditioning regimen, use of serotherapy, prophylaxis and treatment for graft-versus-host disease (GvHD) and viral infections may have a major impact on IR.[3–8] Despite numerous studies, predictive

immune parameters of clinical outcomes remains elusive due to the variety of cut-off values for lymphocyte counts, time post-HSCT for samples collections, and the group of patients included in each study.[4]

There is a dearth of studies regarding IR after pediatric HSCT, especially for patients transplanted in resource-limited countries. In many places around the world, socioeconomic problems, availability of well-matched donors, lack of drugs and tests limit access to this curative procedure. These barriers culminate in a lower volume of HSCT, and consequently in lower number of publications on this topic compared with North America and Europe. [9]

Our objective was to analyze the absolute lymphocyte count (ALC) and lymphocyte subpopulations after HSCT and correlate these factors with transplant characteristics and post-transplant endpoints, such as overall survival, acute GvHD (aGvHD), cytomegalovirus (CMV) infection, and death.

METHODS

This study was conducted at the HSCT Unit of Hospital Pequeno Príncipe in Curitiba, Brazil, one of the largest pediatric HSCT centers in Brazil. Mostly financed by public resources, the center has been performing autologous HSCTs since 2011. Subsequently, allo-HSCT was established for matched sibling donors (MSD) (2013), haploidentical donors (2014), and unrelated donors (2017). This is a retrospective study based on descriptive and quantitative analyses. Data was obtained from the hospital database with prior local Ethics Committee approval (3.318.929).

Patient cohort

From January 2013 to December 2018, 122 patients under 18 years of age underwent allo-HSCT. From this initial group, those who experienced primary graft failure (n=7), received stem cells from umbilical cord blood (n=3) or did not undergo a conditioning regimen (n=1) were excluded. The final cohort included 111 patients. For lymphocyte recovery analysis, patients who experienced secondary graft failure or relapse, those who died before each cut-off day, and those with incomplete history were also excluded.

Conditioning regimen and GvHD immuneprophylaxis

Conditioning regimens consisted of cyclophosphamide + total body irradiation (CY+TBI, n=28), busulfan + fludarabine ± rabbit antithymocyte globulin/alemtuzumab (BU+FLU±ATG/Alemtuzumab, n=22), CY±ATG (n=14), FLU+TBI (n=12), BU+CY (n=10), CY+FLU+TBI±ATG (n=8), CY+FLU±ATG (n=6), BU+FLU + melphalan ± ATG (n=5), other regimens (n=6). A total of 36 patients received serotherapy in conditioning regimen (ATG, n=35; Alemtuzumab, n=1). GVHD prophylaxis was based on cyclosporine + methotrexate (CsA+MTX, n=62, 56%), post-transplant cyclophosphamide + cyclosporine + mycophenolate mofetil (PT-Cy+CsA+MMF, n=31, 28%), CsA+MMF (n=12, 11%), and others (n=6, 5%).

Definitions

The date of stem cell infusion was defined as day zero. Lymphocyte recovery was classified based on the ALC in peripheral blood obtained using complete blood count routinely performed on D+30, D+100 and D+180. Based on previous studies, lymphocyte recovery was defined as ALC[?]300/μL for D+30, ALC[?]500/μL for D+100, and ALC[?]750/μL for D+180.[10–12]

Flow cytometry immunophenotyping of peripheral blood was used to assess CD3+CD4+, CD3+CD8+, CD16+CD56+ and CD19+ counts obtained 100 days after HSCT (±14 days). Recovery of subtypes CD3+CD4+, CD3+CD8+ and CD19+ were classified as absolute counts [?]200/μL and [?]150/μL for CD16+CD56+. Thus, patients with absolute counts below the described values were classified as not recovered based on previous studies.[13,14]

Engraftment was confirmed through an absolute count of neutrophils and platelets in peripheral blood, and the chimerism test using polymerase chain reaction (PCR) short tandem repeat detection of donor DNA. Neutrophil engraftment was considered as the first day of 3 consecutive days when neutrophil count

[?]500/μL, and platelet engraftment was the seventh day with platelet count [?]20 000/μL unsupported by transfusion. [15] CMV infection was screened using pp65 antigenemia or DNA detection by quantitative PCR assay. Acute GvHD was defined using standard clinical and laboratorial criteria and classified as grade 1-4, considering only aGvHD [?]grade 2 as clinically significant.[16] Chronic GvHD was not analyzed due to the reduced number of cases up to D+180. Among hematological malignancies, disease phase was defined as early in case of first or second remission, and advanced in case of third or fourth remission, or active disease at the time of HSCT.

Statistics

We used Pearson's $\chi 2$ qualitative test or Fisher's exact test to analyze the association of lymphocyte recovery with the following categorical independent variables: gender, age group ([?] and <8 years), disease type (malignant, non-malignant), disease phase (early, advanced), donor type (MSD, haploidentical, unrelated), ABO compatibility (compatible, incompatible), stem cell source (peripheral blood stem cells [PBSC], bone marrow [BM]), conditioning regimen (myeloablative conditioning [MAC], reduced-intensity conditioning [RIC]), and use of serotherapy (yes, no). Mann-Whitney U test was used to assess the association of lymphocyte recovery with the dose of total nucleated cells (TNC) and CD34+ infused, and Kruskal-Wallis test to compare lymphocyte counts according to the donor.

Time-dependent variables were calculated from the date of the HSCT to the event. Respective cumulative incidences (CI) were estimated using Gray's method, accounting for competing risks. Death without an event was considered a competing risk for all variables, however, for aGvHD graft failure was also included. To evaluate the effect of significant variables on such outcomes we performed Fine-Gray competing risks univariate and multivariate regression. Overall survival (OS) was analyzed using the Kaplan-Meier method and differences between groups were estimated using log-rank test. Univariate and multivariate Cox regression models were used to assess the impact of the significant variables. Only significant variables in univariate analyses were included in multivariate models. Due to the small sample size, it was not possible to evaluate subgroups of immune recovery for outcomes. All$P$ -values <0.05 were considered statistically significant. Statistical analyses were performed using SPSS version 25.0 (SPSS Inc., Chicago, IL, USA), and EZR version 1.40 (Saitama Medical Center, Jichi Medical University, Japan).

RESULTS

Patient characteristics and clinical outcomes after HSCT are described in Table 1. Although the center is located in the state of Paraná, most patients (59%) were from other Brazilian states, spanning up to 3 400 kilometers.

The cohort consisted of malignant and non-malignant diseases (BM failure syndromes, inborn errors of innate immunity and inborn errors of metabolism). Diagnoses of malignant diseases were acute lymphoblastic leukemia (n=37), acute myeloblastic leukemia (n=10), acute biphenotypic leukemia (n=4), myelodysplastic syndrome (n=2), juvenile myelomonocytic leukemia (n=1) and non-Hodgkin's Lymphoma (n=1). Bone marrow failure syndromes were acquired severe aplastic anemia (n=14), Fanconi anemia (n=11), Blackfan Diamond anemia (n=7) and dyskeratosis congenita (n=2). Inborn errors of innate immunity included severe combined immunodeficiency (n=9), Wiskott Aldrich syndrome (n=5), hemophagocytic lymphohistiocytosis (n=2), CD40L deficiency (n=1) and Griscelli syndrome (n=1). Adrenoleukodystrophy (n=3) and mucopolysaccharidosis type I (n=1) represented the inborn errors of metabolism.

Lymphocyte reconstitution

Serial ALC assessments showed a gradual increase from D+30 to D+180. Median counts reached 634/μL (8-3 133) on D+30, 1 022/μL (133-5 503) on D+100 e 1 541/μL (187-14 432) on D+180, representing 82%, 89% and 90% of recovered patients, respectively. Difference in ALC according to the donor was significant on D+30 (Fig. 1).

Flow cytometry analysis on D+100 showed the highest CD3+CD8+ median count (336/μL, 8-3 569), followed by CD3+CD4+ (154/μL, 8-689) and CD16+CD56+ (130/μL, 14-1 184) while CD19+ had the lowest (56/μL,

0-727). CD19+ had a slow reconstitution and a significant difference according to the donor, as haploidentical HSCT (haplo-HSCT) had a median of 23/μL whereas 69/μL for MSD and 72/μL for unrelated donor (Fig. 2).

Factors associated with lymphocyte recovery

Variables associated with ALC and lymphocyte subpopulations recoveries are described in Table 2. No association was found with sex, status of malignancy, ABO compatibility, or dose of TNC and CD34+ infused. CD3+CD8+, CD16+CD56+ and CD19+ recoveries had no associations with the categorical variables analyzed. CD3+CD8+ recovery had no significant difference according to the use and non-use of serotherapy (54% vs. 75%, P =0.06).

Incidence of clinical complications according to lymphocyte recovery

The 180-days CI of CMV infection was significantly higher for patients with ALC and CD3+CD8+ recovered on D+100 (Table 2). Higher infection rates were also observed in females (57% vs. male 29%,P =0.004), advanced malignancy (56% vs. early 26%,P =0.03), haplo-HSCT (58% vs. MSD 33%, unrelated donor 30%,P =0.02) and with PBSC vs. BM (64% vs. 36%, P =0.01). In the multivariate analysis, ALC<500/μL and CD3+CD8+<200/μL effects on CMV infection remained significant (Table 3).

The 1-year CI of aGvHD [?]grade 2 was higher in haplo-HSCT (24% vs. unrelated donor 9%, MSD 7%, P =0.006), with PBSC (43% vs. BM 9%, P =0.003) and malignant diseases (17% vs. non-malignant 9%,P =0.02). Inadequate CD3+CD4+ recovery was the only immune parameter associated with aGvHD (Table 2). In univariate analysis, this recovery represented a lower risk for the disease occurrence with a Hazard ratio (HR) of 0.23 (0.07-0.76, P =0.02). Multivariate analysis revealed that CD3+CD4+ recovery remained significant when adjusted for the use of PBSC and haplo-HSCT (Table 3).

Lymphocyte recovery and survival

Over a median follow-up of 28 months, 22 patients died between days +22 and +1 778. The causes of death were relapse (n=14), infection (n=6), and GvHD (n=2). Factors associated with higher OS were non-malignant diseases (95% vs. malignant 71%, P <0.001) and BM as stem cell source (87% vs. SP 57%, P <0.001). ALC and CD3+CD4+ recovery on D+100 were the lymphocyte parameters associated with higher survival rates (Fig. 3). In multivariate analyses, ALC recovery on D+100 showed a protective effect on survival if adjusted for malignant diseases, except for BM grafts that did not reach statistical significance (Table 3).

DISCUSSION

This study analyzed lymphocyte recovery after allo-HSCT in pediatric patients with malignant and non-malignant diseases up to 180 days after HSCT. Over this 6-month period, lymphocyte reconstitution was gradual, and each subtype showed different reconstitution rates on D+100. Our study reiterates that lymphocyte count after HSCT impacts patient survival and is associated with pre-transplant factors such as age, source of stem cells, donor, serotherapy, conditioning, disease, and clinical outcomes like CMV and aGvHD. [3,6–8]

On D+100, 89% of patients achieved ALC recovery while CD3+CD8+ and CD16+CD56+ lymphocytes showed the highest recovery rates (68% and 47%, respectively). As natural killer (NK) lymphocytes and TCD8+ clonal expansion propel lymphocyte reconstitution in the first 3 months after HSCT, ALC recovery in our cohort occurred possibly due to these subtypes. [3,17] Kim et al (2013) also obtained this pattern of reconstitution in children and similar median counts for the same period, except for the higher percentage of NK recovered patients owing to a lower cut-off value based on the age-matched reference values. [18]

In this study, patients <8 years showed better recovery for both ALC on D+30 and CD3+CD4+ on D+100. This finding contrast with those of Bartelink et al (2013), which indicated no association between IR and age in a cohort that also included umbilical cord blood transplants.[19] Previous studies have showed an earlier recovery trend in younger patients, through rapid recovery of CD3+CD4+, CD19+ and ALC in patients <5

years and CD3+CD8+, CD19+ and CD16+CD56+ in patients <10 years. [7,18] A consensus on this matter is hampered because published studies have used distinct lymphocyte recovery criteria according to stem cell sources and age-matched reference values. Our results are inconclusive due to the small number of patients. However, there is evidence that children rapidly recover naïve TCD4+ compared with adults, supporting the association between age and greater pre-HSCT thymic activity as an influencing factor in IR. [20]

The potential deleterious effect on thymus caused by MAC regimen was attenuated owing to higher ALC recovery on D+30 when compared with that in the RIC regimen. In children, this impact might be reduced because the thymus is fully functional until puberty, after which thymopoiesis progressively diminishes. [21] Although MAC is related to a better CD19+ recovery compared with RIC regimen in adults, our study showed no association between the type of conditioning regimen and lymphocyte subtype recovery. [22] Patient and transplant heterogeneity led to a variety of preparatory regimens, masking possible correlations between IR and the intensity of these regimens. Moreover, individual conditions (age and weight) may have also altered pharmacokinetics and pharmacodynamics of regimens leading to different exposures and effects on IR. [22,23]

Patients who received BM grafts achieved higher ALC recovery on D+30. Although PBSC mobilized by granulocyte-colony stimulating factor contain more cells contributing to lymphocyte count (CD34+, T lymphocytes and NK) and promote CD3+CD4+ rapid recovery, 93% of the PBSC recipients in this cohort were haplo-HSCT. [24–26] Therefore, donor type may have an impact on early lymphocyte recovery, regardless of the stem cell source.

We observed that serotherapy interfered with ALC recovery on D+180. The same was noted in a pediatric study that indicated inadequate ALC recovery up to 1 year after HSCT. [18] In our cohort, lymphocyte subtypes were not associated with serotherapy. In a large pediatric cohort of malignant and non-malignant diseases, Admiraal et al (2015) reported that only high concentrations of ATG after BM or PBSC transplant reduced TCD4+ reconstitution, whereas in umbilical cord blood transplants even low concentrations of ATG exerted a deleterious effect. Therefore, CD3+CD4+ and serotherapy revealed no association in our study possibly because of the inclusion of only BM and PBSC transplants, the reduced number of patients who used serotherapy, and the inter-patient variability of doses and periods of ATG administration.[27]

Viral infections and lymphocyte reconstitution have a complex correlation as opportunistic infections are related to both the cause and the effect of delayed IR. [28,29] Patients who recovered ALC and CD3+CD8+ on D+100 had a higher CI of CMV infection, which occurred on a median 37 days after transplant in our patients. Literature shows that impairment of CD3+CD8+ early reconstitution contributes to CMV reactivation, while high counts of CD3+CD4+ and CD16+CD56+ represent protective factors in adults.[30,31] But after infection, a clonal expansion of TCD8+ lymphocytes occurs in response to the CMV antigenic stimulus, resulting in an oligoclonal repertoire of memory T cells. [32] The high CD3+CD8+ count observed in patients with CMV infection, described also by Janecsko et al (2016), possibly denotes its specific CMV expansion, which could be confirmed in a larger cohort using molecular tests. [30]

The development of aGvHD [?]grade 2 resulted in lower CD3+CD4+ reconstitution on D+100. Previous reports showed that patients with GvHD had low counts of CD3+CD4+ between the 1st and 3rd months after HSCT, especially naive TCD4+ cells.[33,34] The intense immunodepression is due to the damage that the disease causes in thymic function and BM microenvironment – essential factors for lymphopoiesis – but can be aggravated by GvHD treatment with corticosteroids.[35] Considering the early development of aGvHD in this cohort (median day +28), the poor CD3+CD4+ recovery on D+100 may reflect the disease itself and its treatment.

In our study, haploidentical transplants had a higher CI of CMV infection and aGvHD but OS was similar to other types of transplants. The inadequate ALC recovery on D+30 and the higher CMV infection risk in haplo-HSCT may have resulted from the immunosuppressive effect of GvHD prophylaxis with PT-Cy, CsA and MMF [36–38] We also observed that CD19+ counts on D+100 were lower in haplo-HSCT than in MSD and unrelated transplants. Although major outcomes after haplo-HSCT are comparable to those of

MSD and matched unrelated donors, more studies are needed to better understand IR in the haplo-HSCT settings, especially for pediatric patients with non-malignant diseases[39].

In accordance with a previous study, our data suggest that ALC[?]500/μL on D+100 is a good predictor of OS for pediatric patients undergoing allo-HSCT. [12] Several cell subsets play a crucial role in protecting from infections, however sophisticated exams are not readily available in resource-limited countries. These countries need simple biomarkers to demonstrate if transplanted patients have achieved an acceptable T cell reconstitution. In our study, we observed a trend towards inadequate CD3+CD4+ recovery on D+100 in patients dying from severe infections, but the reduced number of patients was insufficient to reveal a significant effect on OS. However, there is robust evidence in the literature suggesting that this is the most relevant parameter for survival as it is related to mortality from infections.[33,40]

Our retrospective study has important limitations related to the irregularity in immune surveillance by flow cytometry after HSCT. Moreover, the relatively small number of patients and disease heterogeneity did not allow us to reach any definitive conclusions. Nevertheless, these real-world data can be extrapolated to other pediatric HSCT centers and also form the basis for future collaborative studies.

This study exposes the reality in developing countries, ranging from limited financial resources for laboratory tests, to the failure to follow-up due to long travel distances to the reference center. Concurrently, as developed countries move towards predictive medicine in IR monitoring, through greater regularity and specificity of tests, herein we still endeavor to implement cost-effective strategies that may help our patients. To better estimate the time to reach immunocompetence after allo-HSCT in resource-limited countries, we need to standardize IR monitoring with the available tests. Although only studies compiling all data in a prospective way will be able to address this question, monitoring ALC counts on a monthly basis after transplant and measuring lymphocyte subtypes on D+100 and D+180 are easy to perform and could be implemented in clinical practice to identify patients at risk for inadequate IR.

In conclusion, this study summarized the results of IR after HSCT for malignant and non-malignant diseases in one of the main pediatric transplant centers in Latin America. Lymphocyte reconstitution was progressive up to D+180, mainly due to CD3+CD8+ counts. Overall, younger age, BM grafts, MAC regimen, non-malignant disease, non-use of serotherapy, and non-haploidentical donor were associated to adequate lymphocyte recovery. Multiple factors influence IR and our study suggests that ALC[?]500/μL on D+100 may be a predictor of pediatric survival after HSCT, preferably concomitant with lymphocyte subpopulations monitoring.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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LEGENDS

FIGURE 1 Absolute lymphocyte counts according to the type of donor

Absolute lymphocyte count on D+30: matched siblings 746/µL, haploidentical 441/µL and unrelated 616/µL, P =0.03; D+100: matched siblings 1 152/µL, haploidentical 1 066/µL and unrelated 903/µL,P = not significant; D+180: matched siblings 1 714/µL, haploidentical 1 321/µL and unrelated 1 778/µL, P = not significant.

* Statistical significance.

FIGURE 2 Reconstitution of lymphocyte subpopulations on D+100 (n=74)

A: CD3+CD4+ recovery: total (39%); matched siblings (49%), haploidentical (26%) and unrelated (28%), P = not significant.**B:** CD3+CD8+ recovery: total (68%); matched siblings (71%), haploidentical (63%) and unrelated (64%), P = not significant.**C:** CD19+ recovery: total (8%); matched siblings (12%), haploidentical (0%) and unrelated (7%), P = not significant.**D:** CD16+CD56+ recovery: total (47%); matched siblings (39%), haploidentical (53%) and unrelated (64%), P = not significant.

FIGURE 3 Overall survival according to lymphocyte recovery

A : Kaplan-Meier overall survival curve for absolute lymphocyte count recovered patients (blue line) and not recovered (red line) on D+100 and its Hazard Ratio by univariate Cox regression. **B** : Kaplan-Meier overall survival curve and log-rank of CD3+CD4+ recovered patients (blue line) and not recovered (red line) on D+100. Abbreviations: ALC, absolute lymphocyte count; HR, Hazard ratio.

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