Allogeneic haematopoietic stem cell transplantation resets T- and B-cell compartments in sickle cell disease patients

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Abstract

Objectives. Allogeneic haematopoietic stem cell transplantation (allo-HSCT) is the only currently available curative treatment for sickle cell disease (SCD). Here, we comprehensively evaluated the reconstitution of T- and B-cell compartments in 29 SCD patients treated with allo-HSCT and how it correlated with the development of acute graft-versus-host disease (aGvHD).

Methods. T-cell neogenesis was assessed by quantification of signal-joint and β-chain TCR excision circles. B-cell neogenesis was evaluated by quantification of signal-joint and coding-joint K-chain recombination excision circles. T- and B-cell peripheral subset numbers were assessed by flow cytometry. Results. Before allo-HSCT (baseline), T-cell neogenesis was normal in SCD patients compared with age-, gender- and ethnicity-matched healthy controls. Following allo-HSCT, T-cell neogenesis declined but was fully restored to healthy control levels at one year post-transplantation. Peripheral T-cell subset counts were fully restored only at 24 months post-transplantation. Occurrence of acute graft-versus-host disease (aGvHD) transiently affected T- and B-cell neogenesis and overall reconstitution of T- and B-cell peripheral subsets. B-cell neogenesis was significantly higher in SCD patients at baseline than in healthy controls, remaining high throughout the follow-up after allo-HSCT. Notably, after transplantation SCD...
patients showed increased frequencies of IL-10-producing B-regulatory cells and IgM+ memory B-cell subsets compared with baseline levels and with healthy controls. **Conclusion.** Our findings revealed that the T- and B-cell compartments were normally reconstituted in SCD patients after allo-HSCT. In addition, the increase of IL-10-producing B-regulatory cells may contribute to improve immune regulation and homeostasis after transplantation.

**Keywords:** allogeneic haematopoietic stem cell transplantation, B-cell neogenesis, peripheral homeostasis, sickle cell disease, T-cell neogenesis

**INTRODUCTION**

Sickle cell disease (SCD) pathophysiology is marked by complex cellular and molecular interactions involving blood cells, the vascular endothelium and plasma factors, which contribute to vaso-occlusion and consequent tissue injury. Although the innate immune system has been extensively evaluated in this disease, the role of the adaptive immune system is still poorly understood. The available studies report decreased percentages of CD4+ and CD8+ T cells, increased counts of B cell subsets, especially naïve B cells, and impaired B-cell function, including deficient antibody production.

The spleen is the primary organ affected in SCD patients. Multiple infarctions lead to fibrosis and organ atrophy; therefore, virtually all SCD patients present functional asplenia. In general, asplenic patients have immunological impairment and lifelong risk of infections. Splenectomised patients have reduced the numbers of total IgM+ memory B cells and switched B cells, compromising their immunological response against encapsulated pathogens, especially pneumococci. Studies already demonstrate that IgM+IgD CD27+ memory B cells (IgM-only B cells) correspond to < 5% of peripheral blood B cells, while IgM+IgD CD27+ memory B cells carry mutated immunoglobulin variable genes, and these two IgM+ subsets are described as post-germinal centre memory B-cell subsets. However, few studies have addressed these IgM+ memory B cells in SCD patients.

Allogeneic stem cell transplantation (allo-HSCT) is an important curative therapy, currently available for SCD patients. Since the first allo-HSCT in a patient with sickle cell anaemia in 1984, more than 1000 SCD patients have been transplanted worldwide, with favorable outcomes. The success of allo-HSCT is closely related to the quality of the immune reconstitution after transplantation. Thymus and bone marrow functions are noteworthy important in the immune reconstitution and determine clinical outcomes after allo-HSCT. The reinstatement of a diverse T-cell repertoire after allo-HSCT is a continuous and long process, guaranteed by a functional thymus capable of re-establishing the complete T-cell ontogenesis.

Additionally, the B-cell output from the bone marrow plays an important role in the rejuvenation of the adaptive immune system after allo-HSCT in different clinical settings. Particularly, regulatory B cells (Bregs) contribute to maintain the immune tolerance, limit inflammation and re-establish immune homeostasis. Bregs have also been implicated in modulation of immune reactions in transplantation settings mainly by IL-10 secretion.

Little is described about T- and B-cell reconstitution in SCD patients treated after allo-HSCT. We believe that a detailed assessment of the T- and B-cell compartments (neogenesis and peripheral cell subsets) before and after transplantation is essential to understand the quality of the immune reconstitution in chronically inflamed SCD patients, and how it correlates with the clinical outcomes following allo-HSCT. Therefore, here we analysed the T- and B-cell compartments in 29 SCD treated with allo-HSCT. We also evaluated the impact of the acute GvHD (aGvHD) on the T- and B-cell neogenesis and on the peripheral T- and B-cell subsets. Additionally, since B-cell neogenesis was altered in SCD patients at baseline, we further investigated the phenotype and function of Bregs before and after allo-HSCT.
RESULTS

Clinical assessment of SCD patients after allo-HSCT

Clinical characteristics and outcomes of the 29 SCD patients who underwent allo-HSCT are described in Table 1. All patients had a non-detectable spleen on ultrasonography before transplantation.

Although all patients presented fever while in the neutropenic phase of allo-HSCT, only three developed clinically overt infections: two bacterial pneumonias and one catheter-related bacterial infection. Four (13.8%) of the SCD patients had secondary graft failure, 11 (37.9%) developed aGvHD, and 4 (13.8%) developed chronic GvHD (cGvHD) (Table 1). All patients who developed GvHD (acute or chronic) were successfully treated. One patient is deceased. None of the patients presented primary with graft failure.

Thymic function is normalised in SCD patients after allo-HSCT

sjTRECs and β-TREC levels reflect newly egressed T cells from the thymus. During the first six months after transplantation, sjTREC and β-TREC levels significantly decreased when compared to baseline and to healthy individual levels. T-cell neogenesis started to rise between three and six months, reaching complete recovery at 12 months after allo-HSCT (Figure 1a and b; Supplementary tables 1 and 2). The intrathymic thymocyte proliferation rate, estimated by sjTRECs/β-TREC ratio, remained unaltered throughout follow-up (Figure 1c; Supplementary tables 1 and 2).

Reconstitution of the peripheral T-cell compartment after allo-HSCT

SCD patients undergoing allogeneic HSCT experience a lymphopenia phase demonstrated by reduction number of total lymphocytes in the first 3 months compared with pre-transplant (P < 0.05) (Figure 2a and b). CD4+ T-cell counts decreased until 6 months (P < 0.001), while CD8+ T-cell counts were restored and increased at 6 months post-transplantation compared with baseline (Figure 2c). Naïve CD4+ T-cell counts were decreased in the first year after transplantation compared with baseline and healthy individual levels, and were restored only at 24 months post-HSCT (P < 0.05) (Figure 2d). Central memory CD4+ T-cell numbers were diminished in the first 3 months after transplantation and increased at 6 months, reaching baseline and healthy controls levels (Figure 2e). No significant differences were found for central memory CD8+ T-cell counts, except for a decrease at 3 months post-HSCT compared with baseline and healthy controls (P < 0.05) (Figure 2e). The numbers of effector memory and effector CD8+ T cells started to increase after 3 months post-transplantation reaching normal levels at 24 months compared with baseline (Figure 2f and g).

Acute GvHD compromises early recovery of the T-cell compartment after transplantation

SCD patients with aGvHD presented lower levels (P < 0.01) of sjTREC and β-TREC at 6 months after transplantation, compared to those without GvHD (Figure 3a and b). At 12 months post-transplantation, thymic function normalised to healthy control levels in both aGvHD and non-GvHD patient groups (Figure 3a and b).

Analysis of the peripheral T-cell compartment showed decreased naïve and central memory CD4+ T-cell counts in SCD patients with aGvHD compared to healthy controls and SCD patients without GvHD at 1-month post-transplantation (Supplementary figure 1c-e). The numbers of RTEs were lower in SCD patients with or without aGvHD than in healthy controls throughout follow-up. However, there were no differences between patients with or without aGvHD, regarding the thymic output of RTEs (Supplementary figure 1h).

B-cell neogenesis is increased in SCD patients

B-cell neogenesis was evaluated by quantification of sjKREC levels, which reflect the newly generated B cells, and of Cj levels that represent total B cells. The Cj/sjKREC ratio indicates the...
homeostatic proliferation of B cells after migration from the bone marrow, that is division rate in the periphery.38,39

Before transplantation, the bone marrow exportation of newly generated B cells was significantly higher ($P < 0.01$) in the SCD patients than in healthy subjects (Figure 4a; Supplementary table 3). B-cell neogenesis was drastically reduced in the first month ($P < 0.01$) after allo-HSCT, but at 6 months, the amount of sjKREC recovered to baseline levels and was sustained throughout follow-up (Figure 4a; Supplementary table 3). Cj and sjKREC kinetics were very similar (Figure 4b). The mean value of Cj molecules at pre-transplant was not different from healthy controls (Figure 4b, Supplementary table 3). Cj levels were significantly reduced at 1 month after transplantation, and this reduction was sustained throughout follow-up (Figure 4b, Supplementary table 3).
allo-HSCT compared with controls ($P < 0.01$) and with pre-transplant levels ($P < 0.05$), but recovered to baseline levels 6 months ($P > 0.05$) after transplantation.

The B-cell division rate in the periphery was significantly lower at all time periods, when compared to healthy controls ($P < 0.01$) (Figure 4c; Supplementary table 3). These results were confirmed by negative correlation between sjKREC levels and rate of B-cell division ($r_s = -0.87$, $P < 0.0001$) at 12 months after transplantation.

**Reconstitution of the peripheral B-cell compartment after allo-HSCT**

Since SCD patients presented high output of naïve B cells, which did not normalise after allo-HSCT, we sought to evaluate the reconstitution of B-cell subsets and quantify unspecific IgM and IgG levels. Absolute counts of total CD19+ and naïve B cells (CD19+CD27IgD+) reached baseline levels after 6 and 3 months post-transplantation, respectively (Figure 5a and b). Moreover, the numbers of these B-cell subsets were increased at 24 months post-allo-HSCT compared with baseline. These data corroborate the sjKREC and Cj results, evidencing increased generation of naïve B cells in SCD patients at baseline and after transplantation.

In contrast, the decreased numbers of classical memory B-cell subsets (CD19+CD27+ IgD+ unswitched memory and CD19+CD27*IgD+ switch memory) were found at 1, 3 and 6 months after allo-HSCT (Figure 5c and d), which recovered to healthy control levels at 12 months post-transplantation. Moreover, IgD+CD27high plasma cell counts were increased at baseline compared with healthy controls, normalising at 1 month but again increasing to baseline levels from 12 months after allo-HSCT onwards (Figure 5f).

Figure 1. T-cell neogenesis is transiently decreased following transplantation and then normalised after allo-HSCT. Mean ($\pm$ SD) total number of (a) sjTREC and (b) β-TREC values measured by RT-PCR before (pre-transplantation, $n = 19$) and at 1 ($n = 20$), 3 ($n = 20$), 6 ($n = 18$), 12 ($n = 16$), 24 ($n = 12$) and > 24 ($n = 14$) months after allogeneic transplantation (allo-HSCT). (c) Intrathymic T-cell division ($n$) was calculated using the following formula: $n = \log_2$(sjTREC/ β-TREC). Shaded areas indicate mean (dotted lines) and ± SD values for the group of healthy controls ($n = 16$). *Statistical difference between pre- and post-transplantation time points ($P < 0.05$). ϕ Statistical difference comparing all time points (post-transplantation) with those of the healthy control group ($P < 0.05$).
Since IgM⁺ memory B-cell subsets are decreased in patients with functional asplenia,\textsuperscript{21} we decided to quantify them in our SCD patients before and after allo-HSCT. Frequencies of CD19⁺CD27⁺IgM⁺IgD⁺ and CD19⁺CD27⁺IgM⁺IgD⁻ memory B-cell subsets were retrospectively analysed in cryopreserved PBMC from the same SCD patients and were available from two different time points: baseline and 12 months after allo-HSCT (Figure 7). Frequencies of CD19⁺CD27⁺IgM⁺IgD⁺ and CD19⁺CD27⁺IgM⁺IgD⁻ memory B-cell subsets were higher in SCD patients at 12 months after HSCT than in healthy individuals (Figure 7b).

Additionally, we evaluated serum levels of BAFF and APRIL, which are growth factors essential for B-cell homeostasis (Figure 5g and h). Serum BAFF levels were markedly increased during 24 months after allo-HSCT compared with baseline and with control levels (Figure 5g). We found a negative correlation between BAFF/CD19⁺ B-cell ratio and naive B-cell generation (Figure 5i). Throughout follow-up, sjKRECs and Cj correlated with naive and total B cells, respectively (Figure 6a and b). Additionally, BAFF/CD19⁺ B-cell ratio correlated negatively with sjKREC levels (Figure 6c). APRIL concentrations were increased in SCD patients compared with healthy controls. APRIL serum...
levels were highly increased early after transplantation and then returned to baseline levels (Figure 5h).

**Acute GvHD did not affect the recovery of the peripheral B-cell compartment after HSCT**

Additionally, we evaluated whether the development of aGvHD affected the recovery of the B-cell neogenesis and peripheral subsets. Neither sjKREC/Cj levels nor B-cell divisions were different in SCD patients with aGvHD and without GvHD following allo-HSCT (Supplementary figure 3). Therefore, we suggest that B-cell neogenesis is not impaired by the occurrence of aGvHD. Peripheral B-cell subsets presented similar reconstitution dynamics in patients with aGvHD and without GvHD (Supplementary figure 4). Levels of BAFF and APRIL were found to be elevated in patients with aGvHD at 6 months and 1 month, respectively, compared to patients without aGvHD (Supplementary figure 4g and h).

**Modulation of inflammatory cytokine levels after allo-HSCT**

We also evaluated serum levels of TNF-α, IL-8 and IL-18 to understand the inflammatory status after transplantation and its influence on B- and T-cell neogenesis. Serum levels of TNF-α were increased at one and 24 months after allo-HSCT when compared to baseline, and at all time points when compared to the group of healthy individuals ($P < 0.05$) (Supplementary figure 5a). Concentrations of IL-18 were increased at baseline and at one month after transplantation when compared to healthy individuals ($P < 0.05$) and decreased at 3 months. When compared to baseline, IL-18 levels were increased at 24 months after allo-HSCT (Supplementary figure 5b). Serum levels of IL-8 were increased only at one month after allo-HSCT compared with baseline (Supplementary figure 5c).

**Increased frequencies of regulatory and IL-10-producing B cells after allo-HSCT**

Bregs are very important components of the B-cell compartment and modulate immune responses mainly by the production of IL-10.40-43 Frequencies of CD19⁺CD24hiCD38hi transitional Bregs and CD19⁺CD24hiCD27⁺ B10 Bregs were higher at 12 months post-transplantation than at baseline (11% vs. 8.1% and 14% vs. 9%, respectively) albeit not statistically significant (Figure 7b). Figure 7a and c illustrates the gating strategies to analyse regulatory B-cell subsets.

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Figure 3. Acute GvHD leads to a delayed T-cell neogenesis in the first months after transplantation. Mean (± SD) of total number of (a) sjTREC and (b) β-TREC values measured by RT-PCR before the transplant (pre-transplant) and following time points after allogeneic transplantation (allo-HSCT) at 1, 3, 6, 12, 24 and > 24 months in SCD patients who developed aGvHD and in SCD patients who did not develop GvHD. Black lines represent non-GvHD group, and red lines represent the aGvHD group. Statistical analysis was performed using the linear regression mixed model composed of random and fixed effects. *Statistical difference between pre- and post-transplantation time points in the non-GvHD and aGvHD group ($P < 0.05$). For the sjTREC comparison: * aGvHD: pre- vs. 1m and 3m and non-GvHD: pre- vs. 1m and 3m. For the β-TREC comparison: * aGvHD: pre- vs. 6m and non-GvHD: pre- vs. 1m. For the CS comparison: * aGvHD: pre- vs. 24m and non-GvHD: pre- vs. 3m; ** statistical difference between non-GvHD and aGvHD ($P < 0.05$); § statistical difference between aGvHD and the healthy control group ($P < 0.05$); §§ statistical difference between non-GvHD and the healthy control group ($P < 0.05$); other combinations were NS.
Percentages of total CD19+IL-10+ were increased at one year after allo-HSCT compared with baseline (P = 0.03) (Figure 7d) and positively correlated with sjKREC counts (r_s = 0.88, P = 0.01).

We further evaluated IL-10 expression by CD19+CD24hiCD38hi and CD19+CD24hiCD27+ Bregs (Figure 7e). At 12 months post-transplantation, expression of IL-10 by CD19+CD24hiCD38hi Bregs in SCD patients was higher than in healthy controls (P = 0.02), and expression of IL-10 by CD19+CD24hiCD27+ Bregs was higher than at baseline (P = 0.04) (Figure 7e).

DISCUSSION

Allo-HSCT is an established treatment for SCD patients, which mitigates disease manifestations, and is associated with the possibility of cure or long-term improvement of quality of life.30,45 Herein, we evaluated the impact of allo-HSCT on T- and B-cell compartments of SCD patients, since the immunological status has a key role in the success of transplantation.46 We show that SCD patients have increased B-cell neogenesis at baseline compared with healthy controls and that allo-HSCT partially restores these abnormalities.

sjTREC levels are surrogate markers of thymic function, useful for monitoring of the immune reconstitution after allo-HSCT. Low sjTREC counts are associated with poor clinical outcomes and vulnerability to infections.32 Before transplantation, the SCD patients included in this study presented normal sjTREC levels and intrathymic division rates, reflecting normal T-cell neogenesis. Decreased thymic exportation of naïve T cells is expected in the early periods after allo-HSCT, because of the intense myeloablative conditioning regimen and of the delay in thymic recovery.31,46 Indeed, in our patients, sjTREC levels decreased in the first 6 months after allo-HSCT, but normalised within the next 6 months, indicating adequate immune reconstitution.

Young age may have contributed to a favorable recovery of thymic function in our cohort of SCD patients. Patients younger than 25 years usually restore thymic function within the first year after allo-HSCT.47 Conversely, advanced age may negatively affect T-cell neogenesis and intrathymic proliferation.48 Our patients had a median age of 19 years at time of transplantation and received haematopoietic stem cells from younger siblings, which may explain their effective and early thymic

Figure 4. B-cell neogenesis is increased in SCD patients before and after treatment with allo-HSCT. Mean (± SD) of total number of (a) sjKREC and (b) Cj values measured by RT-PCR before the transplant (pre-transplant, n = 19) and following time points after allogeneic transplantation (allo-HSCT) at 1 month and 3 months (n = 20), 6 months (n = 18), 12 months (n = 16), 24 months (n = 12) and > 24 months (n = 14). (c) B-cell division (n) was calculated using the following formula: n = log2(Cj / sjKREC). Shaded areas indicate mean (dotted lines) and ± SD values for the group of healthy controls (n = 16). *P < 0.05 and ɸ P < 0.05 comparing all time point values with those of the healthy control group.
rebound following transplantation. The transient impact of aGvHD on thymic function in those young patients is also consistent with previous data in other allo-HSCT settings.47

One year and 2 years after allo-HSCT, SCD patients still presented systemic inflammation, evidenced by high serum levels of TNF-α and IL-18. In addition, one third of our patients developed aGvHD after transplantation, which has a deleterious impact on T-cell repertoire diversity,47 as well as on the clonal expansion of alloreactive T cells.49 Patients with aGvHD presented a transiently abnormal T-cell neogenesis in the first months after transplantation, in accordance with previous studies.47,50 This suggests a minimal impact of SCD on the thymic microenvironment with no impairment of the thymic regenerative properties in such patients.

In our SCD patients, we found increased B-cell neogenesis that was not reverted after allo-HSCT. B-cell hyperfunction, persisting after surgical splenectomy, has been reported in children with immune thrombocytopenic purpura.51 In this setting, we conjecture that the high output of
naive B cells from the bone marrow is a compensatory mechanism to the decreased numbers of peripheral memory B-cell subsets, especially the IgM+ memory B cells, which have been related to impaired splenic function in SCD patients. The long time after allo-HSCT re-occurrence of some pre-transplant characteristics of the B-cell compartment suggests a persistent impact of the abnormal bone marrow and/or spleen SCD environment on B-cell development and homeostasis in those patients, conversely to our observations in thymic function.

We also speculate that persisting inflammation may affect the haematopoietic progenitor cells, as demonstrated in β-thalassaemia patients, and consequently stimulate B-cell neogenesis in SCD. In fact, bone marrow aspirates from SCD patients showed increased inflammation and aggregation, contributing to significant differences in the quality of haematopoietic stem cell progenitors. Accordingly, we found persistent inflammation, marked by high plasma levels of pro-inflammatory cytokines after transplantation compared with baseline and with healthy individuals. Furthermore, high levels of BAFF and APRIL were found in SCD patients early after transplantation compared with those at baseline. BAFF has a crucial role in the reconstitution of B cells, is a key regulator of B-cell homeostasis and thus might affect B-cell neogenesis in SCD patients. APRIL promotes IL-10 production and regulatory functions of human B cells and can trigger different responses in vitro, including survival and proliferation of human B cells. APRIL also correlates with IL-10 levels in inflamed tissue of arthritis patients, indicating its relevance in immune homeostasis and immunopathology.

In the past few years, regulatory functions of B cells have been described, in addition to their essential roles in antigen presentation, cytokine secretion and antibody production. Regulatory B cells (Bregs) can modulate immune responses mainly by the production of IL-10. The absence of Bregs and/or dysfunctional Bregs can exacerbate inflammatory responses in autoimmune diseases. Here, we demonstrated increased frequencies of IL-10-producing CD19+ cells at 12 months after allo-HSCT, compared with baseline levels. Moreover, we showed higher IL-10 expression by CD19+CD24hiCD38hi and CD19+CD24hiCD27+ regulatory B cells in SCD one year after transplantation compared with baseline and with healthy controls, respectively.

Figure 6. F Correlations of parameters related to B-cell neogenesis and proliferative state. Spearman’s correlation between frequency of (a) naive B cells and sjKREC/150 000 PBMC counts, (b) CD19+ B cells and sj/150 000 PBMC counts and (c) BAFF concentration by CD19+ cells and sjKREC/150 000 PBMC counts before the transplant and following time points after allo-HSCT. Different time points can be identified by different colours. y, years; m, months; pre, pre-transplantation period.
Transitional B cells (CD19<sup>+</sup>CD24<sup>hi</sup>CD38<sup>hi</sup>) are the first B-cell population to be detected in the peripheral blood after allo-HSCT. They are necessary developmental intermediates for the generation of human mature B cells. In the months that follow allo-HSCT, the percentage of transitional B cells progressively decreases, while the frequency of mature naive B-cell increases.
Altogether, our findings indicate that allo-HSCT improves, but not fully restores the B-cell compartment in SCD patients. Regardless of the persistent increase in sjKREC levels after transplantation, the newly produced Bregs are more functional, the absolute numbers of memory B cells increase, and the percentages of IgM+ memory B-cell subsets also rise. The increased IL-10-producing B-regulatory cells after transplantation may be additionally beneficial to SCD patients, possibly modulating the chronic inflammatory state.

We acknowledge here some limitations of this work. First, this study lacked a treatment control and a non-SCD transplanted control group of patients for comparisons. The use of healthy controls did not allow us to assess the relative impact of splenectomy in the T- and B-cell compartments. In fact, to determine whether SCD pathogenesis affects the reconstitution of T- and B-cell compartments after allo-HSCT, we would need an age-matched group of patients with other haematological conditions (with an intact spleen, such as aplastic anaemia) also treated with allo-HSCT. Second, eight of the initial 20 enrolled patients were followed only up to 1 year post-HSCT because of the study inclusion period. Except for one patient who died because of secondary graft failure, we were not able to detect differences in age, sex and immunological status between these patients and those who completed follow-up. Nevertheless, there is a potential risk of bias, concerning the T-cell reconstitution results, which should be acknowledged. In this context, interpretation of early post-transplant T-cell reconstitution is challenging because of the presence of donor T cells infused with the graft and/or residual recipient T cells, before stable mixed chimerism is achieved in the patients. Third, the mechanism of increased B-cell neogenesis in SCD patients at baseline, which persists after allo-HSCT, remains to be explored for the same reason. These issues are of interest for further evaluations on a new controlled study. Finally, most patients with aGVHD were under immunosuppressive treatment with either steroids or other immunosuppressants, which may also have affected the immune reconstitution evaluation.

In conclusion, we demonstrate that the T- and B-cell compartments are normally reconstituted in SCD patients treated with allo-HSCT. The occurrence of aGVHD only transiently affected reconstitution of the adaptive immune system. We also found favourable immunological outcomes of allo-HSCT in SCD that may benefit patients beyond the original purpose of the procedure, such as the increased frequency of IL-10-producing regulatory B cells. This knowledge may be used in future to improve allo-HSCT protocols for SCD patients.

**METHODS**

**Study design**

Twenty-nine SCD patients who underwent HLA-identical sibling allo-HSCT at the Ribeirão Preto Medical School, University of São Paulo, Brazil, were prospectively evaluated for immune reconstitution. The median (range) age was 19 (7–35) years, and 16 (55%) were men. Table 1 describes patient baseline characteristics, clinical outcomes and details about the procedure. A control group consisted of 16 healthy individuals, matched by age/gender/ethnicity with the patients (median age 22.5, range 11–33 years; eight men). This study followed the Good Practice Guidelines and was approved by the local institutional review board (N. 1.011.064/2015 and 1.095.730355/2015). Written consent was obtained from each participant or his/her legal representative at study enrolment. Supplementary figure 6 illustrates the study design.

Graft failure was classified as primary, when patients never achieved absolute neutrophil count > 0.5 x 10⁹ L⁻¹, or as secondary, when patients lost donor chimerism, identified through a qualitative variable number of tandem repeat (VNTR) analysis. Intravenous methotrexate and cyclosporine A were used as GvHD prophylaxis, according to institutional protocol. The presence of GvHD and classification of acute (aGvHD) and chronic GvHD were defined according to the Glucksberg modified score and the National Institutes of Health 2014 criteria, respectively. First-line treatment consisted of corticosteroids (1 mg kg⁻¹ day⁻¹ in moderate/severe chronic GvHD, and 2 mg kg⁻¹ day⁻¹ in GvHD grades II-IV) started immediately upon diagnosis of GvHD. Patients were retrospectively clustered into aGvHD patients who developed acute GvHD, and non-GvHD patients who did not develop acute or chronic GvHD after allo-HSCT. The immunological analyses did not include patients who developed only chronic GvHD because of limited number (N = 4).

Peripheral blood samples were collected from SCD patients prior to (Pre, pre-transplantation, or baseline) and at 1, 3, 6, 12, 24 and > 24 months after transplantation. Patients were evaluated for previous surgical extraction and for size of the spleen, by analysis of medical records and abdominal ultrasound reports, respectively.

**Evaluation of T- and B-cell neogenesis**

Peripheral blood mononuclear cells (PBMCs) were isolated using the Ficoll-Hypaque density gradient centrifugation (GE Healthcare, Chicago, IL, USA). Genomic DNA was
purified from PBMCs using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s recommendations. T-cell neogenesis was assessed by the quantification of signal-joint and \( \beta \)-chain TCR excision circles (sjTRECs and \( \beta \)-TRECs). B-cell neogenesis was determined by quantification of signal-joint and coding-joint K-chain recombination excision circles (sjKRECs and Cj) through real-time quantitative PCR (RTqPCR) (ViiA7 Real-Time PCR System; Applied Biosystems, CA, USA) as previously described.\(^{45,55}\) Multiplex amplification and final quantification of sjTREC, \( \beta \)-TREC, sjKREC and Cj have been previously published.\(^{38}\) Data were validated if at least 50,000 genome equivalents were detected by albumin qPCR, and results were expressed as \( \log_{10} (\text{value}/150.000 \text{ PBMC}).\)

Quantification of T- and B-cell peripheral subsets

Fresh peripheral whole blood samples were immunophenotyped with predetermined optimal antibody concentrations to quantify T- and B-cell peripheral subsets. The following anti-human monoclonal antibodies (mAbs) were used to quantify B-cell subsets: CD19 (HIB19), CD27 (L128) and immunoglobulin D (IgD) (IA6-2) from BD Pharmingen (San Diego, CA, USA). For quantification of T-cell subsets, we used the following mAbs: CD3 (UCHT1), CD4 (RPA-T4), CD8 (RPA-T8), CD45RA (HI100), CD45RO (UCHL1), CD27 (L128) and CD31 (WM59), from BD Pharmingen. Cells were acquired in a FACS Calibur flow cytometer (Becton- Dickinson, San Jose, CA, USA), and data were analysed by the FlowJo (TreeStar, USA) software. Gating strategies are shown (Supplementary figures 6-8), and results are expressed as absolute cell numbers.

Quantification of serum BAFF and APRIL levels

Soluble BAFF and APRIL concentrations were determined by multiplex bead array technology (Magnetic Luminex Assay; R&D System Inc., MN, USA). Plates were read on the Bio-Plex\(\textsuperscript{\textregistered} 200\) platform (Bio-Plex; Bio-Rad Laboratories Inc., CA, USA).

Frequency analyses of IL-10-producing total B cells or B-cell subsets

PBMCs were cryopreserved in 10\% dimethyl sulphoxide and stored at \(-80^\circ\text{C}\) in liquid nitrogen. For the experiments, PBMCs were thawed directly in 37\(^{\circ}\)C heated RPMI 1640 complete medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10\% inactivated human AB serum and 1\% penicillin/streptomycin. Then, the cells were washed in 10X PBS supplemented with 5\% citrate-dextrose solution (Sigma-Aldrich), resuspended in fresh complete RPMI medium and plated in a 96-well U-bottom plate at a concentration of \(1 \times 10^6\) cells ml\(^{-1}\). Analysis of IL-10 production from B cells was evaluated by two in vitro methods upon stimulation with CpG (cytosine guanine dinucleotide [CpG] \( \pm \) CD40L), as previously described.\(^{38}\) After 24 h of incubation, cells were stained for CD19 surface marker and permeabilised IL-10 was detected by anti-IL-10 isotype-matched control. In the same assay, one well with no activated cells was separated for evaluation of the frequency of B-cell subsets using the following anti-human monoclonal antibodies (mAbs): CD19 (HIB19), CD27 (L128), CD38 (HIT2), immunoglobulin M (IgM) (G20-127) and immunoglobulin D (IgD) (IA6-2) from BD Pharmingen (San Diego). Cells were acquired in a FACS Aria II flow cytometer (Becton-Dickinson), and data were analysed by the Flow Jo (TreeStar) software.

Statistical analysis

Data were analysed by the SAS software (SAS Institute Inc. 2008, SAS/STAT\textsuperscript{\textregistered} 9.2 User’s Guide. SAS Institute Inc., Cary, NC, USA) assuming a significance level of 5\% (\( P < 0.05\)). Statistical significance was evaluated longitudinally using the linear regression mixed model composed of random and fixed effects. This model allows multiple longitudinal observations per individual across a baseline period and subsequent time points after transplantation, and applies to the analysis of data on which responses are grouped (more than one measure to the same individual) and the assumption of independence among observations in the same group is not adequate.\(^{54}\) Fixed effects are groups and periods. Random effects are associated with patients since it is necessary to control correlations among repeated measures. For variable frequency, we used a logarithmic transformation to fit data to the proposed model.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Luciana Ribeiro Jarduli Maciel: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Writing – original draft; Writing – review & editing. Julia Teixeira Cottas Azevedo: Data
T- and B-cell neogenesis in SCD patients after HSCT

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