Biol Blood Marrow Transplant 25 (2019) 1260–1268

Biology of Blood and Marrow Transplantation

journal homepage: www.bbmt.org

Analysis

Effect of Graft-versus-Host Disease Prophylaxis Regimens on T and B Cell Reconstitution after Allogeneic Hematopoietic Stem Cell Transplantation

Johan Törlén[1,2,*], Ahmed Gaballa[3], Mats Remberger[2,4,5], Lisa-Mari Mörk[3], Berit Sundberg[2], Jonas Mattsson[2,6], Michael Uhlin[3,6,7]

[1] *Cell Therapy and Allogeneic Stem Cell Transplantation, Karolinska University Hospital, Stockholm, Sweden*
[2] *Department of Oncology-Pathology, Karolinska Institutet, Stockholm, Sweden*
[3] *Department of Clinical Science, Intervention, and Technology, Karolinska Institutet, Stockholm, Sweden*
[4] *Department of Medical Sciences, Uppsala University, Uppsala, Sweden*
[5] *Clinical Research and Development Unit, Uppsala University Hospital, Uppsala, Sweden*
[6] *Department of Immunology and Transfusion Medicine, Karolinska University Hospital, Stockholm, Sweden*
[7] *Department of Applied Physics, Royal Institute of Technology, Stockholm, Sweden*

Article history:
Received 5 October 2018
Accepted 17 January 2019

Keywords:
Allogeneic hematopoietic stem cell transplantation
Immune reconstitution
Clinical trial
GVHD prophylaxis
Lymphocyte excision circles

A B S T R A C T

Lymphocyte reconstitution is pivotal for successful long-term outcome after allogeneic hematopoietic stem cell transplantation (HSCT), and conditioning regimen and post-transplantation immunosuppression are risk factors for prolonged immunodeficiency. Nevertheless, the effects of different immunosuppressive protocols on lymphocyte output and replicative capacity have not been investigated. Here we assessed T cell receptor excision circles (TREC), kappa-deleting recombination excision circles (KREC), and T cell telomere length (TL) as proxy markers for immune reconstitution in patients in a prospective randomized trial comparing graft-versus-host disease (GVHD) prophylaxis after transplantation (cyclosporine/methotrexate versus tacrolimus/sirolimus; n = 200). Results showed that medians of TREC, KREC, and TL were not significantly different between the prophylaxis groups at any assessment time point during follow-up (24 months), but the kinetics of TREC, KREC, and TL were significantly influenced by other transplantation-related factors. Older recipient age, the use of antithymocyte globulin before graft infusion, and use of peripheral blood stem cell grafts were associated with lower TREC levels, whereas acute GVHD transiently affected KREC levels. Patients with lymphocyte excision circle levels above the median at ≤6 months post-transplantation had reduced transplantation-related mortality and superior 5-year overall survival ($P < .05$). We noticed significant T cell telomere shortening in the patient population as a whole during follow-up. Our results suggest that lymphocyte reconstitution after transplantation is not altered by different immunosuppressive protocols. This study has been registered at ClinicalTrials.gov (identifier: NCT00993343).

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) can be curative for a broad spectrum of diseases, predominantly high-risk hematologic malignancies [1]. Pretransplantation conditioning regimens and subsequent immunosuppressive protocols constitute risk factors for long-term humoral and cellular immunodeficiency, contributing to morbidity and mortality by post-transplantation infection, disease relapse, or secondary malignancy [2,3]. To quantitatively and qualitatively restore immune competence, T cell and B cell reconstitution from primary lymphoid organs in the host are pivotal. Previous reports have shown that assessing recipient thymic and bone marrow function can predict HSCT outcome [4,5]. A practical method for monitoring thymopoiesis and B cell reconstitution after transplantation is the quantification of T cell receptor excision circles (TRECs) generated during T cell receptor gene rearrangement and kappa-deleting recombination excision circles (KRECs) generated during Ig light chain rearrangement, respectively [6-8].

An additional requirement for effective immune reconstitution in the host is the capacity of hematopoietic cells to self-renew, which entails telomere shortening at each round of somatic cell division [9]. Shortened telomeres in all cell lineages (including T cells and B cells) have been observed in HSCT

Financial disclosure: See Acknowledgments on page 1267.
Johan Törlén and Ahmed Gaballa share first authorship for this work.
* Correspondence and reprint requests: Johan Törlén, MD, Department of Oncology-Pathology, Karolinska Institute, Karolinska University Hospital, Z1:00, SE-171 76 Stockholm, Sweden.
E-mail address: johan.karlsson.torlen@ki.se (J. Törlén).

https://doi.org/10.1016/j.bbmt.2019.01.029

survivors compared with their donors, especially in the first year after transplantation [10-12]. Short telomeres might limit the cells' replicative capacity, which in theory can lead to premature senescence of immune cells and related complications [10,13]. Although the effects of different transplantation-related factors on lymphocyte output and telomere length (TL) have been studied, the impact of graft-versus-host disease (GVHD) prophylaxis protocols on immune reconstitution has not yet been described. In this study, we analyzed blood samples from a prospective randomized trial comparing cyclosporine and methotrexate (CsA/Mtx) with tacrolimus and sirolimus (Tac/Sir) as GVHD prophylaxis after HSCT, with the aim of assessing TREC and KREC levels and TL between the treatment arms and in relation to long-term outcomes. Clinical results from the trial (ClinicalTrials.gov identifier 00993343), published in 2016, showed clinically comparable transplantation-related outcomes with the 2 GVHD prophylaxis regimens [14]. To the best of our knowledge, TRECs, KRECs, and TL have not been assessed previously in any comparable prospective randomized trial in the field of HSCT.

METHODS

Patients and Samples

All patients (n = 209) included in the prospective randomized trial comparing CsA/Mtx and Tac/Sir as GVHD prophylaxis after HSCT were assessed for inclusion in this subsequent study (Supplementary Figure S1). All patients with at least 1 retrievable blood sample fit for analysis of TRECs, KRECs, and/or telomere kinetics at standardized time points after HSCT (TRECs: 2, 3, 6, 12, and 24 months; KRECs: 2, 3, 6, and 12 months; TL: 3, 12, and 24 months) were included (n = 200). The study was approved by the regional Ethical Review Board in Stockholm (DNR 2016/317-31/3), which previously approved the prospective clinical trial of GVHD prophylaxis (DNR 2006/1430-31/3). A biobank application to collect applicable study samples from the existing HSCT chimerism biobank at the hospital was approved by the Karolinska University biobank unit (BbK-01501). Written informed consent to data retrieval and DNA collection was obtained from each patient (or from parents/guardians of patients age <18 years) before the start of HSCT conditioning. Patient, donor, and transplantation characteristics are listed in Table 1.

Transplantation Procedures

All HSCT recipients and donors were typed for HLA class I and II antigens, using molecular high-resolution typing by polymerase chain reaction (PCR) sequence-specific primers [15]. The intensity and type of conditioning used depended on patient age, diagnosis, and disease stage and was given as myeloablative conditioning (MAC) or reduced-intensity conditioning (RIC) according to standard operating protocols at the center, as described in detail previously [14]. In vivo T cell depletion by administration of antithymocyte globulin (ATG) during conditioning was used in patients receiving grafts from unrelated donors and patients with nonmalignant disorders. A total dose of 4 to 8 mg/kg was used; 4 mg/kg in patients with malignant diseases receiving RIC, 6 mg/kg in patients with malignant diseases receiving MAC, and 8 mg/kg in patients with nonmalignant diseases or receiving HLA-mismatched grafts [16,17]. The graft source was bone marrow or peripheral blood stem cells (PBSCs). Type of GVHD prophylaxis was assigned randomly and consisted of CsA/Mtx or Tac/Sir according to the clinical study protocol [14]. Supportive care followed institutional standards as described previously [18].

Definitions and Outcome Assessment

During standard HSCT follow-up, acute GVHD (aGVHD) was diagnosed and graded according to the Consensus Conference criteria [19], and chronic GVHD (cGVHD) was graded using the National Institutes of Health consensus criteria for clinical trials [20]. Peripheral T cell subset levels at studied time points after HSCT were retrieved from medical records obtained during standardized follow-up. Transplantation-related mortality (TRM) was defined as death from any cause without relapse. Survival time was calculated from the day of transplantation until death or last follow-up. Relapse was defined as recurrent disease after complete remission or disease progression after partial remission or stable disease.

TREC, KREC, and Telomere Quantification

Genomic DNA was extracted from $CD3^+$ and $CD19^+$ beaded blood samples (for TREC and KREC analyses, respectively) using a DNA extraction kit (DiaSorin, Saluggia, Italy) and the NorDiag Arrow system (Isogen Life Science, De Meern, The Netherlands) according to the manufacturers' instructions. Samples were stored at -20 °C until analysis. The δRec-ψJα signal joint TREC and the joint recombination signal sequence intron κ-deleting element (ie, KREC) were quantified separately using a TaqMan real-time PCR. Levels were calculated by the ΔCt method, using the ratio between amplified TREC/KREC and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as described previously [21]. Primer and probe sequences for all reactions are listed in Supplementary Table 1. In brief, a PCR reaction was developed for TREC/GAPDH in a final volume of 25 μL consisting of 12.5 μL of TaqMan 2× Universal PCR Master Mix (Applied Biosystems, Foster City, CA), a final concentration of 300 nM TREC primers and 200 nM probe, and a final concentration of 150 nM GAPDH primers and 100 nM probe. For KREC quantification, KREC primers and probes were used in final concentrations of 900 nM and 200 nM, respectively [6]. To detect GAPDH, the same primer and probe concentrations were used as for TREC reactions. The PCR amplification

Table 1
Patient, Donor, and Transplantation Characteristics by Treatment Arm

Variable	CsA/Mtx	Tac/Sir	*P* Value
Number of patients	103	97	
Age, yr, median (range)	53 (.6-71)	51 (2.8-68)	.64
Age <18 yr, n (%)	12 (12)	13 (13)	.87
Sex, male/female, n (%)	57/46 (55/45)	63/34 (65/35)	.21
Follow-up, yr, median (range)	7.6 (3.9-10.3)	7.2 (3.9-10.3)	.50
Diagnosis, n (%)			
AML/ALL	27/19 (26/18)	22/22 (23/23)	.97
CLL	7 (7)	15 (15)	.08
Lymphoma	14 (14)	13 (13)	.87
MDS	19 (18)	14 (14)	.57
Other malignancies	7 (7)	8 (8)	.90
Nonmalignant	10 (10)	3 (3)	.11
Disease stage, early/late, n (%)	53/50 (51/48)	36/61 (37/63)	.06
Conditioning regimen, n (%)			
MAC/RIC	28/75 (27/73)	28/69 (29/71)	.91
TBI-based	31 (30)	39 (40)	.18
ATG, n (%)	78 (76)	69 (71)	.57
Donor type, n (%)			
Sibling/MUD	28/75 (27/73)	31/66 (32/68)	.56
Donor age, yr, median (range)	28 (4-66)	32 (7-66)	.22
Female donor to male recipient, n (%)	14 (14)	14 (14)	.97
HSCT graft source, n (%)			
BM/PBSCs	21/82 (20/80)	18/79 (19/81)	.88
TNC dose, $\times 10^8$/kg, median (range)	9.3 (1.8-34.0)	11.1 (1.8-42.8)	.10
$CD34^+$ cell dose, $\times 10^6$/kg, median (range)	6.6 (1.2-22.8)	6.3 (1.3-19.7)	.70

CsA, cyclosporine A; Mtx, methotrexate; Tac, tacrolimus; Sir, sirolimus; AML, acute myelogenous leukemia; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; MDS, myelodysplastic syndrome; MAC, myeloablative conditioning; RIC, reduced-intensity conditioning; TBI, total body irradiation; ATG, anti-thymocyte globulin; MUD, matched unrelated donor; HSCT, allogeneic hematopoietic stem cell transplantation; BM, bone marrow; PBSCs, peripheral blood stem cells; TNC, total nucleated cell.

was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The PCR cycling program was as follows: 1 × (95 °C for 10 minutes), 40 × (95 °C for 15 seconds, 60 °C for 1 minute), followed by cooling. Relative average TL was measured using a similar PCR-based telomere assay. In brief, telomere DNA repeats were amplified by PCR, and the telomere amplification product was set in relation to the single-copy gene β-globulin, according to methods described previously [22].

Statistical Analysis

Patient characteristics are presented as absolute and relative frequencies. Because the kinetics of lymphocyte excision circles and telomeres can vary substantially among patients, results are presented and statistically analyzed in 10-log values if applicable. Categorical variables were compared using the chi-square test, and continuous variables were compared using the Mann-Whitney *U* test. Relationships between given variables were investigated using the nonparametric test of Spearman's rank correlation coefficient. The cumulative proportion surviving was calculated using the Kaplan-Meier method and compared using the log-rank test. The incidence of TRM was investigated using an estimator of cumulative incidence curves. Patients were censored at the time of death or last follow-up. For multivariate analysis, logistic regression was used to evaluate factors affecting TREC, KREC, and telomeres. Levels of TREC and KRECs and TL at the different time points were dichotomized at median values. Levels over the median were considered high. Factors with a *P* value <.10 in the univariate analysis were included in the backward elimination multivariate analysis; factors analyzed included patient age, sex, diagnosis, disease stage, donor type, sex match, year of HSCT, ATG use, GVHD prophylaxis, stem cell source, total nucleated cell dose, and $CD34^+$ cell dose. A 2-sided *P* value <.05 was considered statistically significant for all tests. All analyses were performed using Statistica software (StatSoft, Tulsa, OK) and SPSS Statistics (IBM, Armonk, NY).

RESULTS

Patient Characteristics

Clinical results from the prospective randomized trial have been published previously [14]. At least 1 analyzable blood sample from set time points during HSCT follow-up could be retrieved for 103 patients in the CsA/Mtx arm and for 97 patients in the Tac/Sir arm (Supplementary Table 2). No statistically significant differences were detected between the prophylaxis groups for important patient or donor characteristics (Table 1). Median follow-up of the GVHD prophylaxis cohorts was 7.6 years in the CsA/Mtx arm and 7.2 years in the Tac/Sir arm at the time of statistical analyses.

Impact of GVHD Prophylaxis on TREC and KREC Levels and TL

The median TREC and KREC levels and TL were similar in the 2 GVHD prophylaxis groups at all assessed time points after HSCT (Figure 1A-C). In addition, there were no differences in levels of any of the major T cell subsets ($CD3^+$, $CD4^+$, or $CD8^+$) in peripheral blood between the 2 GVHD prophylaxis groups at 3, 6, 12, or 24 months after HSCT (Supplementary Table 3).

TREC Kinetics Post-HSCT

Overall, there was a gradual increase in TREC frequency in the study population during the duration of the trial, with statistically significant differences between medians at 6 and 12 months after HSCT ($P < .001$) (Supplementary Figure S2A). Younger recipient age, defined as ≤40 years at the time of HSCT, correlated significantly with higher TREC levels at all time points ($P < .005$) (Figure 2A). Multivariate analysis showed significant correlations between higher TREC levels at 12 months (odds ratio [OR], .96, 95% confidence interval [CI], .94 to .98; $P < .001$), and 24 months (OR, .92; 95% CI, .89 to .96; $P < .0001$) post-HSCT with younger recipient age (Table 2). Other factors with a significant impact on TREC levels after HSCT was ATG-treatment and stem cell source (Figure 2B and C). The use of ATG-containing conditioning regimens was associated with significantly lower TREC levels at early time points post-HSCT (OR at 2 months, .24 [$P < .001$]; OR at 3 months, .13 [$P < .0001$]; OR at 6 months, .34 [$P < .01$]), with differences

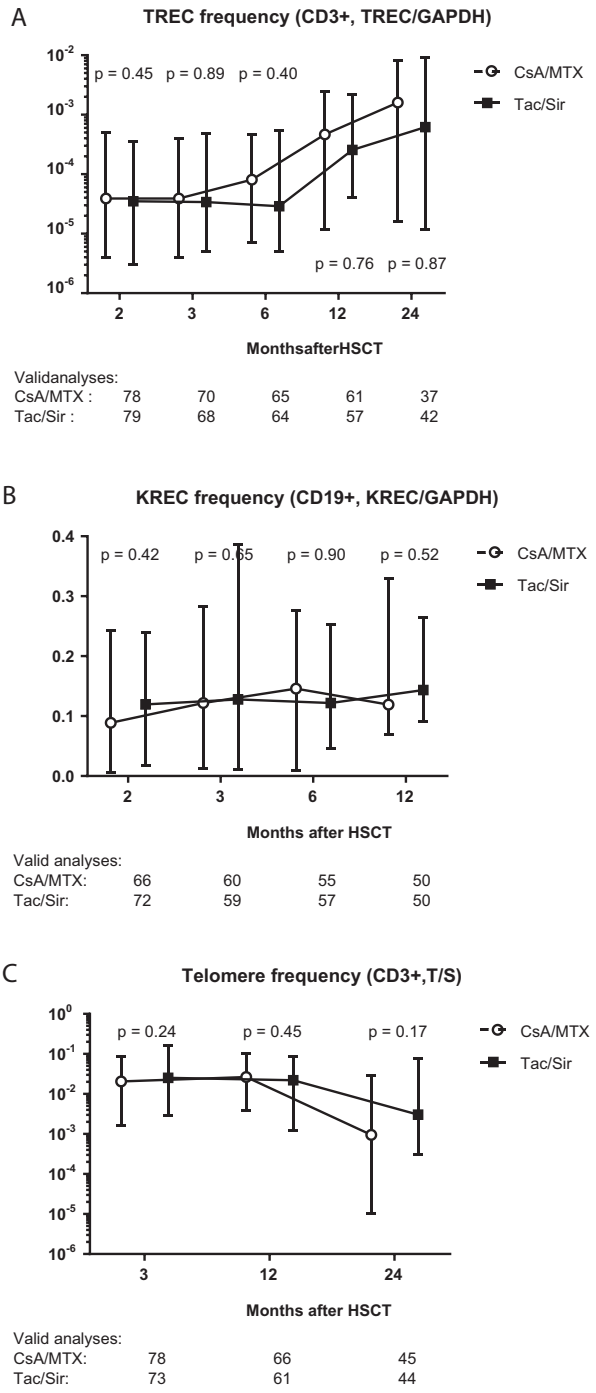

Figure 1. Kinetics of excision circles at different time points after HSCT. (A) Median TREC levels with whiskers for upper/lower quartiles, according to treatment arm. (B) Median KREC levels with whiskers for upper/lower quartiles, according to treatment arm. (C) Median telomere levels with whiskers for upper/lower quartiles, according to treatment arm. Datasets are interleaved at each time point to improve readability. T, telomere amplification product; S, single-copy gene (β-globulin).

disappearing at 12 months post-HSCT. There was no significant difference in TREC levels between the GVHD prophylactic groups during follow-up, irrespective of the use of ATG in conditioning (Supplementary Figure S3). Compared with recipients of PBSC grafts, recipients of bone marrow grafts had higher TREC levels at 12 months ($P < .05$) and 24 months ($P < .01$) post-HSCT. Regarding conditioning regimen intensity,

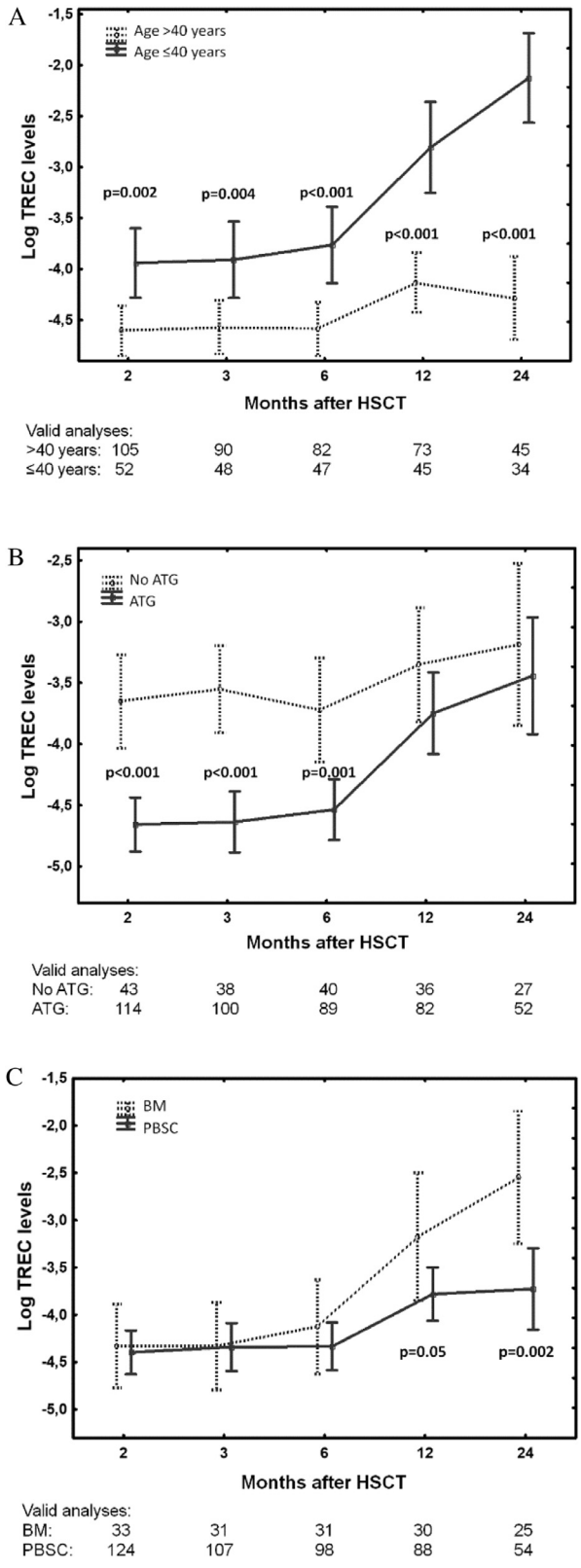

Figure 2. Impacts of transplantation factors on TREC levels after HSCT. (A) Impact of patient age. (B) Impact of ATG treatment. (C) Impact of graft source. Datasets are interleaved at each time point to improve readability. BM, bone marrow.

significantly higher TREC levels were seen in patients receiving MAC compared with those receiving RIC ($P < .05$ at all time points) (Supplementary Figure S4). Acute GVHD did not affect TREC levels, but patients diagnosed with moderate/severe cGVHD had lower TREC levels at 12 months post-HSCT compared with patients without cGVHD or with mild cGVHD ($P < .05$, data not shown).

KREC Kinetics Post-HSCT

Median KREC levels increased significantly during follow-up, with higher frequencies in samples taken at 12 months compared with those taken at 2 months post-HSCT ($P = .035$) (Supplementary Figure S2B). The sole factor with a significant impact on KREC levels was aGVHD grade II-IV (Figure 3), which was associated with lower levels ≤6 months post-HSCT in affected patients ($P < .05$). This decrease persisted in multivariate analysis at 2 months (OR, .46; 95% CI, .23 to .92; $P = .03$) and 3 months (OR, .44; 95% CI, .20 to .94; $P = .03$) post-HSCT (Table 2). Chronic GVHD of any severity did not affect KREC kinetics during follow-up. Conditioning intensity (MAC versus RIC) and ATG treatment before HSCT did not have a significant impact on KREC levels at any assessed time point (data not shown).

TL Post-HSCT

In the study population as a whole, there was a significant decrease in median TL between 3 months and 24 months post-HSCT ($P = .002$) (Figure 4). Patient age did not affect TL, but multivariate analysis showed a correlation between female sex and lower TL (OR, .43; 95% CI, .20 to .94; $P < .05$) and a correlation between RIC and higher TL (OR, 2.82; 95% CI, 1.19 to 6.65; $P < .05$) at 12 months post-HSCT (Table 2). Treatment with ATG, graft source (bone marrow versus PBSCs), aGVHD or cGVHD, or subsequent relapse after HSCT had no significant impact on TL at any assessed time point (data not shown).

TREC and KREC Levels and HSCT Outcome

To test whether TREC or KREC levels could predict clinical outcome after HSCT, we investigated how levels at different time points correlated with 5-year TRM and overall survival (OS). Patients were divided into 2 groups for each assessed time point, with TREC (or KREC) levels either above or below the calculated median at each time point. For TRECs, patients with levels above the median at 6 months post-HSCT had a significantly lower TRM compared with patients with levels below the median (3.1% versus 15.4%; $P = .04$) (Figure 5A). This effect persisted in survival analysis, with superior 5-year OS in patients with TREC levels above the median compared with patients with levels below the median at 6 months (84% versus 68%; $P = .04$) (Figure 5B). Patients with TREC levels above the median also had significantly less use of ATG ($P < .01$) and greater us of sibling donors ($P = .02$) (Supplementary Table 4). Six patients with TREC levels below the median died of infection during follow-up, compared with no patients with levels above the median ($P = .03$) (Supplementary Table 4).

For KRECs, long-term TRM was significantly lower in patients with levels above the median patients compared with patients with levels below the median at 3 months post-HSCT (8.5% versus 21.7%; $P = .035$) (Figure 5C). There was also a significant difference in 5-year OS between the 2 groups, with superior OS at 3 months post-HSCT in the patients with KREC levels above the median (81% versus 60%; $P = .01$) (Figure 5D). Patients with KREC levels above the median at 3 months had more favorable disease stages at time of HSCT ($P = .02$), and fewer received conditioning protocols containing total body irradiation ($P = .04$) (Supplementary Table 5). Nine patients with KREC levels below the median died of infection during follow-up, compared with 2 patients with levels above the median ($P = .05$) (Supplementary

Table 2
Multivariate Analyses of Study Outcomes

Outcome/Factor	OR/HR*	95% CI	*P* Value
TREC level			
2 mo			
ATG	.24	.10-.53	<.001
RIC	.33	.15-.72	<.01
3 mo			
ATG	.13	.05-.33	<.001
6 mo			
ATG	.34	.16-.76	<.01
12 mo			
Age ≤40 yr	.96	.94-.98	<.001
24 mo			
Age ≤40 yr	.92	.89-.96	<.0001
KREC level			
2 mo			
aGVHD grade II-IV	.46	.23-.92	<.05
3 mo			
aGVHD grade II-IV	.44	.20-.94	<.05
6 mo		No significant factors in multivariate analysis	
12 mo		No significant factors in multivariate analysis	
Telomere level			
3 mo		No significant factors in multivariate analysis	
12 mo			
Female sex	.43	.20-.94	<.05
RIC	2.82	1.19-6.65	<.05
24 mo		No significant factors in multivariate analysis	
Mortality			
TREC > median, 6 mo	.53	.26-1.12	.10
KREC > median, 3 mo	.42	.21-.86	.017
TRM			
TREC > median, 6 mo	.30	.08-1.18	.08
KREC > median, 3 mo	.37	.13-1.03	.056

HR indicates hazard ratio.
* OR for TREC level, KREC level, and telomere level; HR for mortality and TRM.

Table 5). Relapse incidence was similar in the 2 patient groups dichotomized by median TREC or KREC levels, but patients diagnosed with relapse during the trial had significantly lower TREC levels at 2 months ($P = .02$) and lower KREC levels at 6 months post-HSCT ($P < .05$) compared with relapse-free patients (data not shown).

DISCUSSION

In this study, based on a prospective randomized trial comparing CsA/Mtx and Tac/Sir as GVHD prophylaxis after HSCT, we assessed TREC and KREC levels as well as TL to determine how their respective kinetics are affected by transplantation-related factors and how they influence long-term outcomes.

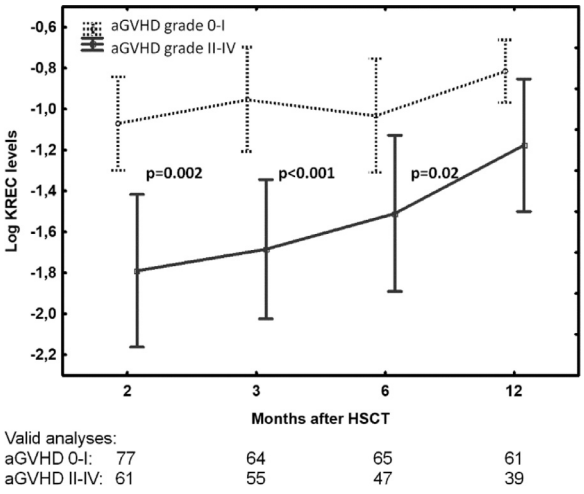

Figure 3. Impact of aGVHD on KREC kinetics at different time points after HSCT. Datasets are interleaved at each time point to improve readability.

The type of GVHD prophylaxis did not affect TREC or KREC levels at any studied post-HSCT time point. Calcineurin inhibitors (CsA and Tac) achieve their immunosuppressive action primarily by preventing dephosphorylation of the nuclear factor of activated T cells, reducing activity of IL-2 coding genes and related cytokines [23]. Sirolimus exerts lymphocyte suppression by receptor-dependent signal transduction mechanisms through the mTOR complex, blocking activation of T and B cells [24]. According to our data, none of the studied immunosuppressive protocols specifically affected (or differed in their eventual effect on) excision circle levels after HSCT, suggesting that similar outcomes of lymphocyte output can be expected irrespective of the GVHD prophylaxis administered after transplantation. Any unforeseen difference between the regimens might require a prolonged pharmacologic exposure to be detectable, but none of the participants remained on Sir treatment for >6 months (median, 68 days of treatment), which we note as a relative study limitation.

When evaluating TREC levels in the whole study cohort, we identified significantly increased levels after 6 months post-HSCT, in agreement with previous publications [7,21]. The adaptive, lymphoid compartment of the immune system reconstitutes slowly after HSCT with risks of long-standing deficits in global immunity [25]. Early expansion of mature T cells in the graft is believed to form a limited repertoire during the first year post-HSCT. This is followed by increased thymus-dependent development of naïve T cells, a process naturally affected by older age, and other factors impairing thymopoiesis

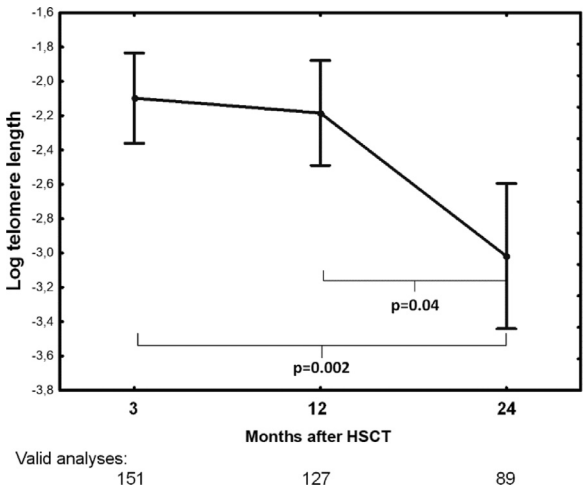

Figure 4. Kinetics of telomeres at different time points after HSCT (all patients).

[21,26]. In this study, recipient age was the strongest determinant for TREC recovery after HSCT, confirming previous studies [21,27,28]. The dominant age factor may explain the higher TREC levels in recipients of MAC compared with recipients of RIC in our study, reflecting the inherent age bias present in the analyses of conditioning intensity groups. This was verified in a subgroup analysis in which conditioning intensity did not remain significant when adjusted for age, because RIC was used primarily in older patients in the trial according to HSCT standards [17,29].

During conditioning, more than 70% of the patients received ATG, to reduce GVHD and the risk of rejection, by in vivo T cell depletion [30]. ATG can be measured in recipients' blood up to 5 weeks after infusion, despite a half-life of only 2 to 3 days [31]. Patients receiving ATG had significantly lower TREC levels up to 6 months post-HSCT, implying a risk for impaired T cell development and slower adaptive immune reconstitution for a considerable period after transplantation. Accordingly, it is recommended that factors related to immune reconstitution be considered in weighing the use (and dose) of ATG in individualized HSCT conditioning protocols.

Numerous studies have compared the use of bone marrow and PBSC grafts and their correlation with HSCT outcomes. In general, PBSC grafts result in higher cell doses and faster engraftment [32]. PBSC grafts are also associated with higher rates of cGVHD compared with bone marrow grafts, and thus PBSCs may be preferred in patients with malignant diseases to decrease the risk of relapse by a graft-versus-leukemia effect [33,34]. In this study, patients receiving bone marrow had significantly higher TREC levels at 12 and 24 months post-HSCT compared with PBSC recipients, suggesting that the graft

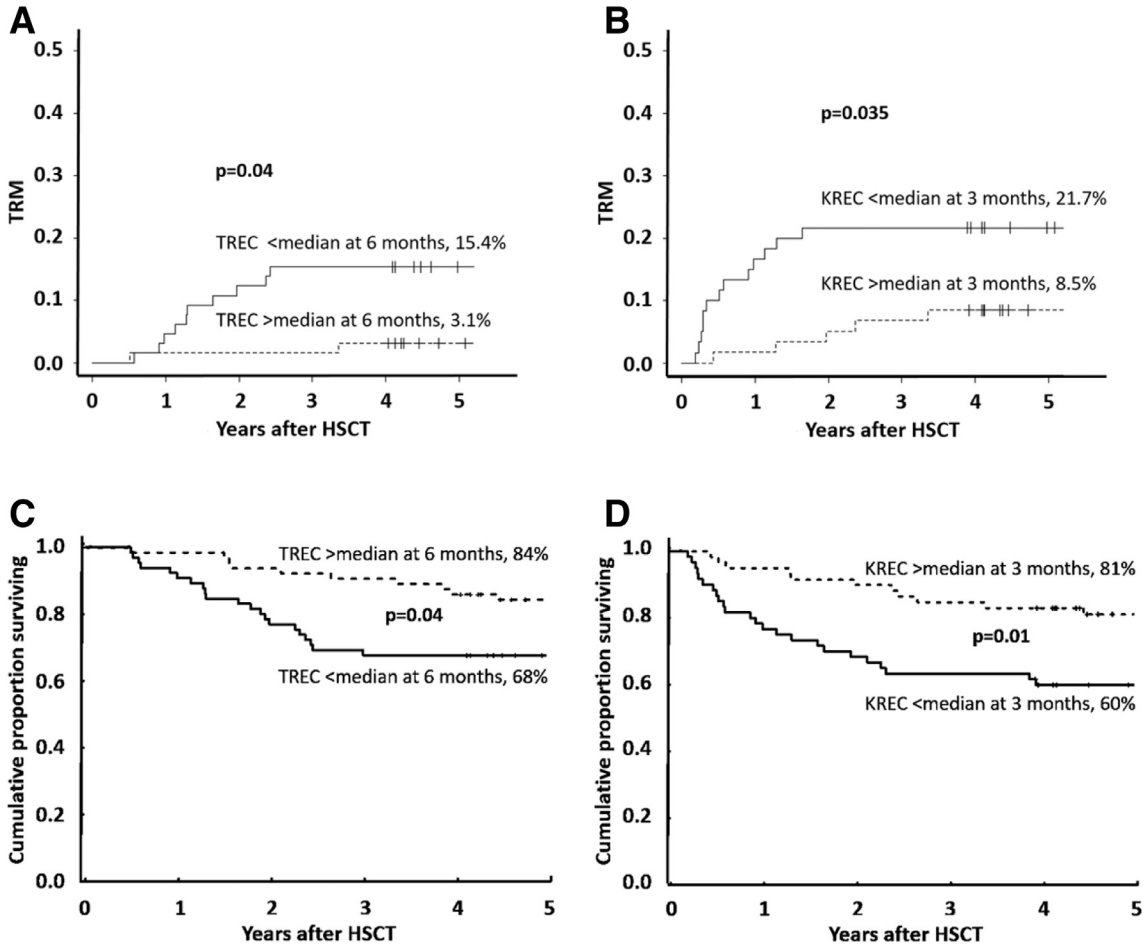

Figure 5. Impact of median excision circle levels on transplantation outcomes. (A) TREC levels above/below median, TRM. (B) KREC levels above/below median, TRM. (C) TREC levels above/below median, OS. (D) KREC levels above/below median, OS.

source may affect late adaptive immune reconstitution. After 1 year, TREC levels also correlated significantly with higher numbers of $CD4^+$ T cells, which are crucial for instigating and shaping adaptive immune responses in the host. The differences in TREC kinetics at later time points could be related to a more diverse and supportive cell population in bone marrow grafts, which contain mesenchymal stromal cells and dendritic cells that can possibly engraft in the host after HSCT [35]. A diverse graft may aid restoration of an advantageous thymic microenvironment in the host and enhance proper T cell selection and output. This effect possibly may be reinforced at later time points after transplantation when immunosuppression is tapered and/or discontinued in patients without GVHD, a condition that can impair immune reconstitution [3]. Regarding the impact of graft source in the analyses, another potential explanation (ie, confounding factor) for higher TREC numbers in patients receiving bone marrow compared with those receiving PBSC could be a younger recipient age in the former group. In general, pediatric patients are more likely to receive bone marrow grafts, and in our study population only 3 patients age <20 years received PBSC grafts. Because younger patients are expected to have better thymic function compared with older patients, graft source may be a surrogate marker of age in this analysis. No cord blood graft recipients were included in our study, but a retrospective study of TREC and KREC levels in HSCT recipients by Nakatani et al [36] suggested that cord blood provided more rapid B cell recovery compared with bone marrow or PBSC grafts.

The only factor significantly affecting KREC kinetics in this study (resulting in lower levels) was aGVHD grade II-IV, consistently found in multivariate analyses at 2 months and 3 months post-HSCT. This finding aligns with published data showing significantly decreased KREC and B cell numbers in HSCT recipients before the development of both aGVHD and cGVHD [7], and may indicate a transient effect of aGVHD on KREC levels. aGVHD did not affect TREC kinetics in our study, but patients diagnosed with moderate/severe cGVHD had lower TREC levels at 12 months post-HSCT. Thus, lower KREC levels coincided with the onset period of classic aGVHD, whereas lower TREC levels were associated with a period of increased incidence of cGVHD. It was previously shown that hematopoietic dysfunction can be exacerbated during GVHD [37-39], and hematopoietic cells express both class I and class II HLA proteins, which represent potential targets for direct immunocompetent cell responses. Mensen et al [40] reported that T cell bone marrow infiltration during aGVHD may be associated with delayed B cell recovery and function after HSCT, and a toxic bone marrow microenvironment impairing donor-derived hematopoiesis (eg, B cell output) also could result from GVHD-related cytokine release [41]. The finding of an association between moderate/severe cGVHD and lower TREC levels in our study likely reflects the thymus itself as a target of cGVHD, resulting in impaired function and T cell production in affected patients [42]. The literature also shows that dynamic imbalances in recovery of T cell subsets after HSCT favor the production of effector T cells over $CD4^+$ regulatory T cells, which can contribute to the development of cGVHD [43].

We identified cutoffs in median TREC and KREC levels that had a significant impact on TRM and 5-year OS after HSCT. These findings indicate that assessment of lymphocyte excision circles is valuable for monitoring immune reconstitution after transplantation, but these findings must be considered in relation to other HSCT parameters affecting TRM and survival outcomes. Impaired T cell and B cell reconstitution are risk factors for viral and fungal infections, and patients with lymphocyte excision circles below the median in our study population succumbed more often to infection complications during follow-up. Thus, prolonged or enhanced infection prophylaxis, faster taper of immunosuppression (in the absence of GVHD), or booster infusion of donor cells to enhance immune reconstitution in select patient groups may be justified if our findings can be confirmed in additional studies. Data to support this reasoning is lacking in the literature, but some studies have demonstrated patterns of late-onset infections in HSCT recipients and have suggested that differences in immune reconstitution cannot be ruled out as a contributing factor [44].

TL over time was similar in our 2 GVHD prophylaxis groups. To our knowledge, similar assessments have not been published previously; However, a relevant study of the effect of different immunosuppressive drugs on lymphocyte TL in healthy individuals was published by Welzl et al in 2014 [45]. Their in vitro results showed that CsA and Tac had a more pronounced prosenescence effect compared with rapamycin after short-term immunosuppressive treatment, with significantly less reduction of TL in peripheral blood mononuclear cells cultured in the presence of rapamycin. If these results of mTOR inhibition are extrapolated to our patient cohort, such effects likely can be ruled out, because the patients randomized to rapamycin treatment also received a calcineurin inhibitor (Tac/Sir).

In accordance with other studies, we found a significant decrease in TL over time in our HSCT recipients. Owing to study limitations, we were unable to investigate this finding in relation to TL in the patients' respective donors. Nevertheless, previous studies have shown that allogeneic HSCT recipients experience severe erosion of blood cell TL after transplantation, possibly due to massive differentiation pressure on cells during repopulation of the host [10-12]. We also report a more prominent reduction of TL in females at 12 months post-HSCT that diminished after 24 months. Chemotherapy-induced injury to granulosa cells can lead to insufficient estrogen production after HSCT [46]. Thus, the shorter TL in females might be a consequence of insufficient telomerase activity in the first year post-HSCT owing to deficient estrogen up-regulation [47], a function feasibly recovered by homeostasis (or estrogen treatment) at later time points after HSCT. This suggested hypothesis is not supported by our present data and should be addressed in future work. We did not measure estrogen levels or telomerase activity in this study, which we identify as a limitation. Studies involving androgenic blockade to improve immune reconstitution (and thymic output) post HSCT are currently ongoing, supported by previous observations that onset of puberty initiates thymic involution [48].

We acknowledge some design limitations of this study, including the use of PCR as the sole method of assessing TREC and KREC levels and telomere kinetics. In addition, the study was performed in a relatively small patient cohort with notable patient- and transplantation-related variations, which might impair or promote differences between study groups. Consequently, these results need to be verified in additional prospective trials and should be viewed primarily as hypothesis-generating when designing future multicenter trials with larger patient populations.

In summary, our results support the use of TREC and KREC assessment as valid parameters to consider during post-HSCT follow-up. Considering this as a proxy marker for qualitative adaptive immune reconstitution, prolonged infection prophylaxis may be warranted in patients with low TREC/KREC levels. Currently available standardized assays for simultaneous TREC and KREC quantification can be readily adapted for clinical use,

complementing analyses performed during follow-up after HSCT. Their usefulness as tools for immunologic monitoring after HSCT may be enhanced in combination with additional immunologic and clinical parameters. Further research is needed to validate and optimize the use and interpretation of lymphocyte excision circles and telomere kinetics after HSCT.

ACKNOWLEDGMENTS

The authors thank the medical, nursing, and laboratory staff of the patient area (PO) Cell Therapy and Allogeneic Stem Cell Transplantation, PO Hematology, and PO Children with Diseases of the Abdomen and Blood and Cancer at the Karolinska University Hospital for their invaluable contributions to the study through their compassionate care of the patients.

Financial disclosure: This research was funded by grants from the Swedish Cancer Society (CAN 2016/747), the Swedish Childhood Cancer Fund (BCF PR2015-0120, 2016-2017 and BCF PR2017-0016, 2018), the Swedish Research Council (VR 2016-00516), and by grants provided by Stockholm County Council (ALF-Project).

Conflict of interest statement: There are no conflict of interest to report.

Authorship statement:J.T., J.M., and M.U. designed the study; J.T., B.S., and L-M.M. collected study samples; J.T., A.G., and B.S. performed laboratory analyses; J.T. and A.G. performed data management and analyses; M.R. performed statistical analyses; J.T. and A.G. drafted the manuscript; and M.R., L-M.M., B.S., J.M., and M.U. reviewed and approved the final manuscript. J.T. and A.G. contributed equally to this work.

SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at doi:10.1016/j.bbmt.2019.01.029.

REFERENCES

1. Majhail NS, Farnia SH, Carpenter PA, et al. Indications for Autologous and Allogeneic Hematopoietic Cell Transplantation: Guidelines from the American Society for Blood and Marrow Transplantation. *Biol Blood Marrow Transplant*. 2015;21:1863–1869.
2. Mackall C, Fry T, Gress R, et al. Background to hematopoietic cell transplantation, including post transplant immune recovery. *Bone Marrow Transplant*. 2009;44:457–462.
3. van den Brink MR, Velardi E, Perales MA. Immune reconstitution following stem cell transplantation. *Hematology Am Soc Hematol Educ Program*. 2015;2015:215–219.
4. Clave E, Rocha V, Talvensaari K, et al. Prognostic value of pretransplantation host thymic function in HLA-identical sibling hematopoietic stem cell transplantation. *Blood*. 2005;105:2608–2613.
5. Corre E, Carmagnat M, Busson M, et al. Long-term immune deficiency after allogeneic stem cell transplantation: B-cell deficiency is associated with late infections. *Haematologica*. 2010;95:1025–1029.
6. Sottini A, Ghidini C, Zanotti C, et al. Simultaneous quantification of recent thymic T-cell and bone marrow B-cell emigrants in patients with primary immunodeficiency undergone to stem cell transplantation. *Clin Immunol*. 2010;136:217–227.
7. Mensen A, Ochs C, Stroux A, et al. Utilization of TREC and KREC quantification for the monitoring of early T- and B-cell neogenesis in adult patients after allogeneic hematopoietic stem cell transplantation. *J Transl Med*. 2013;11:188.
8. Gaballa A, Sundin M, Stikvoort A, et al. T cell receptor excision circle (TREC) monitoring after allogeneic stem cell transplantation; a predictive marker for complications and clinical outcome. *Int J Mol Sci*. 2016;17:E1705.
9. Ruella M, Rocci A, Ricca I, et al. Comparative assessment of telomere length before and after hematopoietic SCT: role of grafted cells in determining post-transplant telomere status. *Bone Marrow Transplant*. 2010;45:505–512.
10. Baerlocher GM, Rovó A, Müller A, et al. Cellular senescence of white blood cells in very-long-term survivors after allogeneic hematopoietic stem cell transplantation: the role of chronic graft-versus-host disease and female donor sex. *Blood*. 2009;114:219–222.
11. Rufer N, Brümmendorf TH, Chapuis B, Helg C, Lansdorp PM, Roosnek E. Accelerated telomere shortening in hematological lineages is limited to the first year following stem cell transplantation. *Blood*. 2001;97:575–577.
12. Akiyama M, Asai O, Kuraishi Y, et al. Shortening of telomeres in recipients of both autologous and allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant*. 2000;25:441–447.
13. de Pauw ES, Otto SA, Wijnen JT, et al. Long-term follow-up of recipients of allogeneic bone marrow grafts reveals no progressive telomere shortening and provides no evidence for haematopoietic stem cell exhaustion. *Br J Haematol*. 2002;116:491–496.
14. Törlen J, Ringdén O, Garming-Legert K, et al. A prospective randomized trial comparing cyclosporine/methotrexate and tacrolimus/sirolimus as graft-versus-host disease prophylaxis after allogeneic hematopoietic stem cell transplantation. *Haematologica*. 2016;101:1417–1425.
15. Olerup O, Zetterquist H. HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: an alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. *Tissue Antigens*. 1992;39:225–235.
16. Remberger M, Svahn BM, Hentschke P, Löfgren C, Ringdén O. Effect on cytokine release and graft-versus-host disease of different anti-T cell antibodies during conditioning for unrelated haematopoietic stem cell transplantation. *Bone Marrow Transplant*. 1999;24:823–830.
17. Slavin S, Nagler A, Naparstek E, et al. Nonmyeloablative stem cell transplantation and cell therapy as an alternative to conventional bone marrow transplantation with lethal cytoreduction for the treatment of malignant and nonmalignant hematologic diseases. *Blood*. 1998;91:756–763.
18. Forslöw U, Mattsson J, Ringden O, Klominek J, Remberger M. Decreasing mortality rate in early pneumonia following hematopoietic stem cell transplantation. *Scand J Infect Dis*. 2006;38:970–976.
19. Przepiorka D, Weisdorf D, Martin P, et al. 1994 Consensus Conference on Acute GVHD Grading. *Bone Marrow Transplant*.. 1995;15:825–828.
20. Filipovich AH, Weisdorf D, Pavletic S, et al. National Institutes of Health consensus development project on criteria for clinical trials in chronic graft-versus-host disease, I: Diagnosis and Staging Working Group Report. *Biol Blood Marrow Transplant*. 2005;11:945–956.
21. Sairafi D, Mattsson J, Uhlin M, Uzunel M. Thymic function after allogeneic stem cell transplantation is dependent on graft source and predictive of long-term survival. *Clin Immunol*. 2012;142:343–350.
22. McGrath M, Wong JY, Michaud D, Hunter DJ, De Vivo I. Telomere length, cigarette smoking, and bladder cancer risk in men and women. *Cancer Epidemiol Biomarkers Prev*. 2007;16:815–819.
23. Wiederrecht G, Lam E, Hung S, Martin M, Sigal N. The mechanism of action of FK-506 and cyclosporin A. *Ann N Y Acad Sci*. 1993;696:9–19.
24. Li J, Kim SG, Blenis J. Rapamycin: one drug, many effects. *Cell Metab*. 2014;19:373–379.
25. Maris M, Boeckh M, Storer B, et al. Immunologic recovery after hematopoietic cell transplantation with nonmyeloablative conditioning. *Exp Hematol*. 2003;31:941–952.
26. Gress RE, Emerson SG, Drobyski WR. Immune reconstitution: how it should work, what's broken, and why it matters. *Biol Blood Marrow Transplant*. 2010;16(1 Suppl):S133–S137.
27. Fallen PR, McGreavey L, Madrigal JA, et al. Factors affecting reconstitution of the T cell compartment in allogeneic haematopoietic cell transplant recipients. *Bone Marrow Transplant*. 2003;32:1001–1014.
28. Douek DC, Vescio RA, Betts MR, et al. Assessment of thymic output in adults after haematopoietic stem-cell transplantation and prediction of T-cell reconstitution. *Lancet*. 2000;355:1875–1881.
29. Martino R, Caballero MD, Canals C, et al. Reduced-intensity conditioning reduces the risk of severe infections after allogeneic peripheral blood stem cell transplantation. *Bone Marrow Transplant*. 2001;28:341–347.
30. Arai Y, Jo T, Matsui H, Kondo T, Takaori-Kondo A. Efficacy of antithymocyte globulin for allogeneic hematopoietic cell transplantation: a systematic review and meta-analysis. *Leuk Lymphoma*. 2017;58:1840–1848.
31. Remberger M, Sundberg B. Rabbit-immunoglobulin G levels in patients receiving thymoglobulin as part of conditioning before unrelated donor stem cell transplantation. *Haematologica*. 2005;90:931–938.
32. Holtick U, Albrecht M, Chemnitz JM, et al. Bone marrow versus peripheral blood allogeneic haematopoietic stem cell transplantation for haematological malignancies in adults. *Cochrane Database Syst Rev*. 2014;4 CD010189.
33. Horowitz MM, Gale RP, Sondel PM, et al. Graft-versus-leukemia reactions after bone marrow transplantation. *Blood*. 1990;75:555–562.
34. Wu S, Zhang C, Zhang X, Xu YQ, Deng TX. Is peripheral blood or bone marrow a better source of stem cells for transplantation in cases of HLA-matched unrelated donors? A meta-analysis. *Crit Rev Oncol Hematol*. 2015;96:20–33.
35. Villaron EM, Almeida J, López-Holgado N, et al. Mesenchymal stem cells are present in peripheral blood and can engraft after allogeneic hematopoietic stem cell transplantation. *Haematologica*. 2004;89:1421–1427.
36. Nakatani K, Imai K, Shigeno M, et al. Cord blood transplantation is associated with rapid B-cell neogenesis compared with BM transplantation. *Bone Marrow Transplant*. 2014;49:1155–1161.
37. Martínez-Jaramillo G, Gómez-Morales E, Sánchez-Valle E, Mayani H. Severe hematopoietic alterations in vitro, in bone marrow transplant recipients who develop graft-versus-host disease. *J Hematother Stem Cell Res*. 2001;10:347–354.

38. Welniak LA, Blazar BR, Murphy WJ. Immunobiology of allogeneic hematopoietic stem cell transplantation. *Annu Rev Immunol*. 2007;25:139–170.
39. von Bonin M, Bornhäuser M. Concise review: the bone marrow niche as a target of graft-versus-host disease. *Stem Cells*. 2014;32:1420–1428.
40. Mensen A, Jöhrens K, Anagnostopoulos I, et al. Bone marrow T-cell infiltration during acute GVHD is associated with delayed B-cell recovery and function after HSCT. *Blood*. 2014;124:963–972.
41. Reddy P, Ferrara JLM. Mouse models of graft-versus-host disease. *StemBook*. Cambridge, MA: Harvard Stem Cell Institute; 2008.
42. Krenger W, Holländer GA. The thymus in GVHD pathophysiology. *Best Pract Res Clin Haematol*. 2008;21:119–128.
43. Alho AC, Kim HT, Chammas MJ, et al. Unbalanced recovery of regulatory and effector T cells after allogeneic stem cell transplantation contributes to chronic GVHD. *Blood*. 2016;127:646–657.
44. Anderson D, DeFor T, Burns L, et al. A comparison of related donor peripheral blood and bone marrow transplants: importance of late-onset chronic graft-versus-host disease and infections. *Biol Blood Marrow Transplant*. 2003;9:52–59.
45. Welzl K, Kern G, Mayer G, et al. Effect of different immunosuppressive drugs on immune cells from young and old healthy persons. *Gerontology*. 2014;60:229–238.
46. Shanis D, Merideth M, Pulanic TK, Savani BN, Battiwalla M, Stratton P. Female long-term survivors after allogeneic hematopoietic stem cell transplantation: evaluation and management. *Semin Hematol*. 2012;49:83–93.
47. Calado RT, Yewdell WT, Wilkerson KL, et al. Sex hormones, acting on the *TERT* gene, increase telomerase activity in human primary hematopoietic cells. *Blood*. 2009;114:2236–2243.
48. Leposavić G, Perisić M. Age-associated remodeling of thymopoiesis: role for gonadal hormones and catecholamines. *Neuroimmunomodulation*. 2008;15:290–322.