T-cell reconstitution after allogeneic stem cell transplantation: assessment by measurement of the sjTREC/βTREC ratio and thymic naïve T cells

Simone Ringhoffer,[1] Markus Rojewski,[2,3] Hartmut Döhner,[1] Donald Bunjes,[1]* and Mark Ringhoffer[1]*

[1]Department of Internal Medicine III, University of Ulm, [2]Institute for Clinical Transfusion Medicine and Immunogenetics, German Red Cross Blood Transfusion Service, Baden-Wuerttemberg-Hessen, Ulm and [3]Institute of Transfusion Medicine, University of Ulm, Germany

ABSTRACT

The immune reconstitution after allogeneic hematopoietic stem cell transplantation comprises thymus-dependent and thymus-independent pathways. We wanted to improve the understanding of this complex process using two different measurements at definite checkpoints of T-cell neogenesis. We therefore assessed the thymus-dependent pathway by combining measurements of single joint T-cell receptor excision circles (sjTREC) and β T-cell receptor excision circles (βTREC) in an improved quantitative light-cycler hybridization polymerase chain reaction assay. In a subgroup of patients, we additionally assessed the proliferation kinetics of the CD31$^+$ thymic naïve cell population, which corresponds to recent thymic emigrants by six-color immunostaining. After the establishment of normal values in 22 healthy volunteers, we applied our polymerase chain reaction to 66 patients undergoing allogeneic hematopoietic stem cell transplantation at a median age of 44 years. It took more than 2 years after transplant to restore the pre-transplant thymic proliferation capacity. Only one third of the patients in our longitudinal study reached age-adjusted normal values for both sjTREC and βTREC at a median follow-up of 558 days, with acute graft-*versus*-host disease being the most prominent negative factor by univariate analysis. We observed several patterns of sjTREC and βTREC recovery suggesting different mechanisms of thymic damage in individual patients. In a comparison of CD31$^+$ thymic naïve cells between volunteers and patients after transplant we found a significantly higher peak proliferation rate within the latter population in the first year after transplantation. The combination of measurements of sjTREC and βTREC by our simplified polymerase chain reaction assay provides insight about the stage of T-cell development affected by different types of damage and may help to choose the correct therapeutic intervention. Besides the sole thymic T-cell neogenesis, proliferation within the CD31$^+$ thymic naïve cell compartment contributed to the replenishment of the naïve T-cell pool after transplantation.

Introduction

In recipients of an allogeneic bone marrow or peripheral stem cell graft, T-cell recovery is crucial to regain immune competence.[1,2] The re-establishment of a diverse T-cell repertoire is essential to provide protection against opportunistic infections and augments host defenses against the recurrence of malignant disease.[3] Mouse models have shown that T-cell reconstitution after an allogeneic stem cell transplant is a biphasic process.[2] In the early phase, T cells derived from the graft expand due to homeostatic proliferation. The T cells derived from homeostatic expansion have a skewed T-cell receptor repertoire and the diversity observed is dependent on the size of the initial T-cell inoculum.[4,5] In a subsequent phase, the thymus resumes T-cell neogenesis and this provides the host with donor-derived naïve T cells with a completely diverse T-cell receptor repertoire.

Most of our knowledge about T-cell development in the steady state and under the conditions of experimental bone marrow transplantation has been gained in the murine system. These studies have demonstrated that T-cell neogenesis is a complex, multi-step process comprising the homing of T-cell progenitors to the thymus, T lineage commitment, rearrangement of the β- and α-T-cell receptor chains, positive selection, negative selection, proliferation and differentiation, export of recent thymic emigrants (RTE) and further maturation and expansion of RTE in stromal niches of peripheral lymphoid organs.[6] The number of RTE exported to the periphery is determined by the amount of proliferation which occurs between β- and α-chain rearrangement.[7,8]

Normal human T-cell development appears to be broadly similar.[9,10] The information about T-cell neogenesis after stem cell transplantation in humans is much more limited because peripheral blood is the only readily accessible compartment for study and this blood contains only 2% of the body's T cells. The most commonly used approach to evaluate thymic activity is to determine the number of naïve T cells in the peripheral blood by flow cytometry. These studies have essentially confirmed the work in mice showing that T-cell reconstitution after transplantation comprises a

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Correspondence: mark.ringhoffer@klinikum-karlsruhe.de

thymus-independent pathway and a thymus-dependent pathway.[11,12] The main limitation of this approach is that it does not actually measure RTE but rather the more mature naïve T cells. In recent years, more refined immunophenotypic approaches have been developed using six- and seven-color flow cytometric analysis to determine the co-expression of CD31 and/or PTK7 on naïve $CD4^+$ T cells as a marker for RTE.[13,14] These approaches have led to the identification of two subpopulations of naïve $CD4^+$ T cells, the $CD4^+CD45RA^+CD31^+$ thymic naïve and the $CD4^+CD45RA^+CD31^-$ central naïve population.[14,15] The thymic naïve population or possibly its $PTK7^+$ subpopulation, appears to represent the population of RTE in humans.[13] These markers have, however, only been applied to a very limited extent in the setting of stem cell transplantation.[16,17] Over the past 10 years, many groups have used the quantification of single-joint (sj) T-cell receptor excision circles (sjTREC) by quantitative polymerase chain reaction (QT-PCR) in various subpopulations in the peripheral blood as a surrogate markers for RTE.[18-22] sjTREC are formed during the rearrangement of the α-chain of the T-cell receptor in the thymus. This approach has proved to be valuable but it is limited by the fact that lymphocyte proliferation induced by antigen and/or cytokines results in a dilution of sjTREC since they are not replicated in the cell cycle.[23] The most informative approach currently available is probably the simultaneous measurement of sjTREC and βTREC by QT-PCR and the determination of the sjTREC/βTREC ratio (thymic factor, TF).[24,25] This method was pioneered by a Canadian-French group. The sjTREC/βTREC ratio reflects the intensity of proliferation between DβJβ rearrangement at the $CD34^+CD38^+CD1^-$ stage and the VαJα rearrangement at the $CD4^+CD8^+$ stage of human thymocyte development which in turn is the major determinant of the number of RTE produced.[7,25] The major advantages of this technology are: (i) the TF is independent of peripheral proliferation; (ii) it measures the thymic neogenesis of all αβ-T cells independently of their phenotype; (iii) it provides information about T-cell receptor diversity; and (iv) since it measures events at two different checkpoints in T-cell development, it may help to localize the stage of development affected by different interventions.

This powerful methodology has so far been applied mainly in the setting of infection by human immunodeficiency virus and to only approximately 50 patients post-transplantation, mainly because it is very labor intensive.[7,25,26] In this study, we applied a simplified method for determining the TF in a cross-sectional and longitudinal study of a large cohort of patients prior to and after allogeneic stem cell transplantation. We supplemented this indirect evaluation of intrathymic events by an investigation of RTE in the early post-thymic phase using six-color, two-laser technology to identify RTE by CD31 expression. These studies provided evidence of different patterns of thymic damage after transplantation, and for the existence of feedback mechanisms between the pool of available RTE and T-cell neogenesis in the thymus.

Methods

Patients

All patients received an allogeneic stem cell or bone marrow transplant between 2004 and 2008 at the bone marrow transplantation unit of the University of Ulm. The study was approved by the local ethics committee of Ulm University. All patients gave their informed consent to participation in the study. An overview of the patients' characteristics is shown in Table 1. More information about the donor types is given in the *Online Supplementary Material*.

Table 1. Overview of the patients' characteristics.

Underlying disease	N.	Age (years)	Status at transplant	T-cell depletion CliniMACS/Campath	MA	RIC	DLI
Acute myeloid leukemia	32	44 (26-59)	CR1/2: 26 RD: 4 PR: 2	Yes: 19 No: 13	26	6	8 (12)
Acute lymphoblastic leukemia	10	38 (18-63)	CR 1-3: 8 PR: 2	Yes: 4 No: 6	8	2	1 (1)
Chronic myeloid leukemia	8	41 (30-57)	CP: 2 CCyR: 6	Yes: 6 No: 2	2	6	4 (8)
Myelodysplastic syndrome	3	48 (44-56)	RAEB NT 2 RCMD NT 1	Yes:2 No:1	3	0	0
Multiple myeloma	3	59 (52-61)	CR: 2 PR: 1	Yes: 0 No: 3	0	3	2 (6)
Lymphoma	5	42 (25-68)	CR: 3 PR: 2	Yes:4 No:1	1	4	0
Chronic myeloproliferative disorder	2	45 (39-50)	Active, NT: 2	Yes:0 No:2	1	1	0
Paroxysmal nocturnal hemoglobinuria	3	22 (20-29)	Active, NT: 3	Yes:3 (1 ALG) No:0	3	0	0
Total	**66**	**44**	**NA**	**TCD: 38/66**	**44**	**22**	**15 (27)**

The age at transplant is given as median and range. CR: complete remission; PR: partial remission; RD: refractory disease; CP: chronic phase; CCyR: complete cytogenetic remission; RAEB: refractory anemia with an excess of blasts; RCMD: refractory cytopenia with multilineage dysplasia; NT,: not treated; Active: active disease; ALG: anti-lymphocyte-globulin; MA: myeloablative conditioning; RIC: reduced intensity conditioning; DLI: donor lymphocyte infusion, given as number of patients who received DLI and as total number of DLI (in brackets).

Supportive care

Patients were treated in single rooms with reverse isolation. Irradiated erythrocyte and platelet concentrates were transfused if the hemoglobin level dropped below 8 g/dL or the platelet count below 20×10^9/L, respectively. Cytomegalovirus-seronegative blood donors were used if both the stem cell donor and recipient were cytomegalovirus-seronegative. All patients were given prophylactic levofloxacin, itraconazole or posaconazole, aciclovir, and cotrimoxazole. Itraconazole or posaconazole, aciclovir, and cotrimoxazole were continued until patients had achieved $CD4^+$ T-cell counts higher than 0.20×10^9/L. Pre-emptive intravenous therapy with ganciclovir 2×5 mg/kg daily, foscarnet 2×60 mg/kg daily or cidofovir 5 mg/kg once a week was initiated if patients became positive in the cytomegalovirus antigenemia test.

Diagnosis and treatment of graft-versus-host disease

The diagnosis of acute and chronic graft-*versus*-host disease (GvHD) was made using established clinical and histopathological criteria.[27,28] More information about GvHD prophylaxis is provided in the *Online Supplementary Material*.

Determination of sjTREC and βTREC

Three different control vectors were constructed: these contained a CD3 genomic sequence plus either a genomic sjTREC or a Dβ1 or Dβ2 consensus sequence. Briefly, TREC were quantified in $CD3^+$ cells, sorted by magnetic separation from peripheral blood mononuclear cells. Approximately 100 ng total DNA was used for the first round multiplex TREC analysis. Primers specific for the sjTREC, each of the ten DβJβ (Dβ1-Jβ1.1-1.6 and Dβ2-Jβ2.1-2.4) and the CD3γ -chain were defined on the human germline sequence as described by Poulin and Dion.[7,25] To generate standard curves, the amplicons for the sjTREC, for a common sequence of all six Dβ1Jβ1 TREC, as well as for the four amplifiable Dβ2Jβ2 TREC were individually cloned into pCR 2.1-TOPO vector (Invitrogen) together with the CD3γ amplification product. The vector construction, the generation of the standards and the TREC quantification are described in more detail in the *Online Supplementary Material*.

Measurement of thymic naïve cells by immunophenotyping

We used a six-color immunophenotyping subgating strategy and primarily identified the T helper cells by co-expression of CD3 and CD4. These $CD3^+CD4^+$ cells were subjected to a $CD45RA^+$ *versus* CD45RO plot. At this stage we set three different gates to separate distinct populations, in a way similar to that published by S. Junge *et al.*[17] Each of the high expressing $CD45RA^+$ cell populations (R2 and R3) was then examined for $CD31^+$ expression and for Ki-67 expression in the respective subset. Our gating strategy is shown in Figure 1.

Statistical methods

The statistical analysis for the influence of transplant-related factors – age, T-cell depletion, intensity of the conditioning, total body irradiation and GvHD (comprising contingency tables with two-sided Fisher's exact test) – was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego,

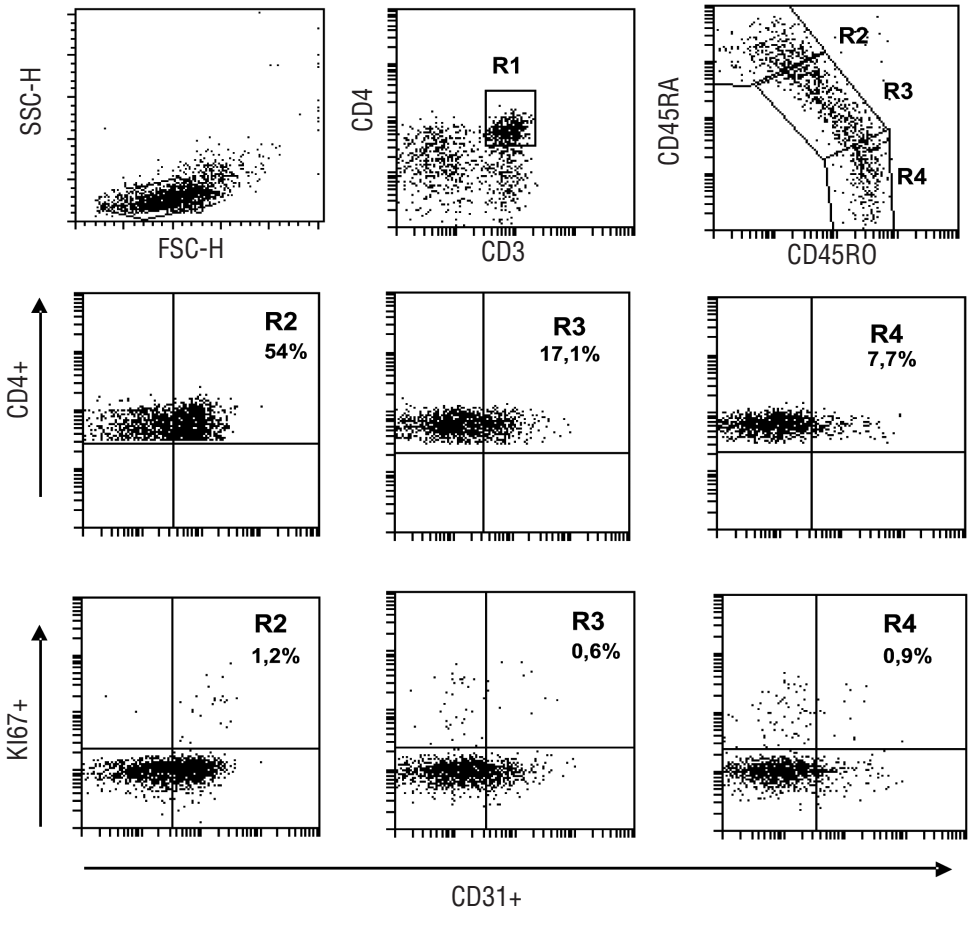

Figure 1. Gating strategy for the determination of $CD3^+CD4^+CD45^+CD31^+Ki67^+$ cells. After a first gating on the $CD3^+CD4^+$ cell population, these cells were divided into three subpopulations depending on their expression of CD45RA and CD45RO. These fractions were examined for the expression of CD31 and the R2 fraction, as the highest CD31-expressing cell population, was then finally subjected to analysis of Ki67 expression as a marker of proliferation.

CA, USA). The Spearman's correlation coefficients and the t-test were calculated with the same program. Additionally, we used Microsoft Excel spreadsheets to construct the regression lines (Seattle, WA, USA). The term physiological reconstitution was introduced to describe the achievement of age-adjusted normal sjTREC and βTREC values as well as a normal TF during the post-transplantation course.

Results

Thymic factor in healthy volunteers and the determination of age-adjusted normal values for sjTREC and βTREC

We first evaluated our assay in a group of 22 healthy volunteers with an age range of 18 to 92 years. The normal values ranged between 36 - 5986 copies (mean: 1421) for sjTREC and between 1 and 47 copies for βTREC (mean: 11) per 10^5 $CD3^+$ cells, resulting in a mean TF of 129. When all patients were included there was a correlation with age for both types of TREC with Spearman's correlation coefficients of -0.93 and -0.79, respectively (Figure 2). This correlation enabled us to construct negative regression lines and to produce two formulas ($y=8618.7e^{-0,0617x}$ for sjTREC and $y=77.408e^{-0,059x}$ for βTREC) in order to determine age-adjusted normal values. The calculated values led to a TF (sj/β-TREC) close to 100, which is described to be normal in healthy young adults.[24] For example, the age-adjusted normal values for sjTREC for a 20- and a 40-year old individual were 2509 and 730 copies per 10^5 $CD3^+$ cells and 24 and 8 for βTREC (TF 105 and 91), respectively. These data suggest that the thymic production capacity for RTE falls by approximately 0.7 % per year. Figure 2C shows that up to the age of 55 years there is an age-dependent decline of the sjTREC without a significant change of βTREC, as previously described.[25,26] In a second step we analyzed seven healthy volunteers aged 70 to 92 years (median age, 77.5 years) and found very low numbers of βTREC in this cohort. An unpaired t-test to compare the βTREC values of the younger and older groups of healthy volunteers showed a statistically significant difference (P=0.0019). Since we could not determine the exact starting point of the decline in βTREC, we assumed an age-related regression curve including all age groups.

Cross-sectional study: development of sjTREC and βTREC after transplantation

Next, we applied this PCR technique to patients' samples and included 226 samples obtained from 66 patients (median age, 44 years) in a cross-sectional study. The samples were collected over a period of more than 2 years after allogeneic transplantation. Patients were heterogeneous with respect to diagnosis and conditioning regimen employed. Briefly, about two-thirds of the patients received standard conditioning and one-third received reduced-intensity conditioning. Thirty-eight of 66 patients received a T-cell-depleted graft, which was depleted by either alemtuzumab or *ex-vivo* $CD34^+$ selection with a CliniMACS device. The median number of transplanted $CD34^+$ cells was 6.75×10^6/kg of body weight. Twenty-seven donor lymphocyte infusions were given to 15 patients. Detailed information is given in Table 1.

These patients' samples were analyzed for their content of sjTREC and βTREC; all values were normalized to 10^5 $CD3^+$ cells. No significant alteration of βTREC production was detected during the observation period. The mean pre-transplant value was seven copies per 10^5 $CD3^+$ cells and the highest mean value after transplantation was six copies per 10^5 $CD3^+$ cells. In contrast, we found a drastically reduced production of sjTREC immediately after transplant (36 copies/10^5 $CD3^+$ cells), which recovered very slowly with time: it took more than 2 years for the sjTREC values to reach their pre-transplant level. Thus, the sjTREC/βTREC ratio (TF) was also severely impaired after transplantation and the mean pre-transplant ratio of 137 was not yet reached after 2 years with a mean value

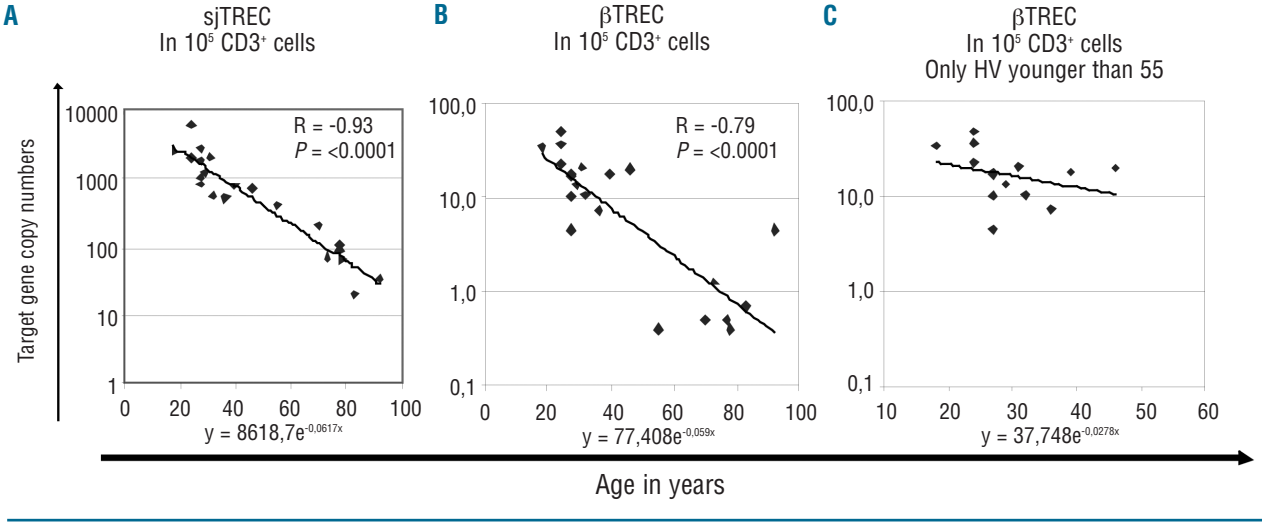

Figure 2. Age dependency of sjTREC and βTREC. Correlation of age and copy number of the target gene in healthy volunteers (HV). The age of the HV ranged from 18 to 92 with a median age of 34 years. There was a negative correlation with age for sjTREC (r=-0.93) (A) as well as for βTREC (r=-0.79) (B) by Spearman's analysis. In (C) we only included HV up to the age of 55 years and found a regression trend with a slower decline.

of 96 (Figure 3). The same was also true if age-adjusted normal values for the whole cohort were used. We calculated age-adjusted normal values of 932 copies for sjTREC, 9 copies/10^5 $CD3^+$ cells for the β-TREC and a TF of 103 for this cohort of patients. Higher age had an impact on the recovery of the TF: only 20% of patients older than 44 years (median age of cohort), reached the pre-transplant TF level 1 year after transplant compared with 45% of the younger group.

TREC production is already reduced before the transplant procedure

Since we found a big difference between the calculated target values of age-matched healthy volunteers and the patients, we wanted to know how much of the thymic damage was present prior therapy. We, therefore, evaluated pre-transplant TREC production and compared it with the previously determined age-adjusted normal values. Thirty-two patients were assessed for sjTREC and βTREC. We found a great variation of pre-transplant TREC levels for both sjTREC and βTREC. Overall, 25/32 (78%) of our patients had lower TREC levels compared to those of healthy adults of the same age. The absolute copy numbers ranged from 1 to 2178 for sjTREC and from 0 to 37 for βTREC. In a comparison between the observed TREC copy numbers and age-adjusted normal values, the median impairment was -62% for sjTREC, -63% for βTREC and -46% for TF. The impact of prior therapy was higher in patients below the median age of 47 years with a median impairment of the TF being -58%. Five of the six patients with the lowest numbers of sjTREC were suffering from acute lymphoblastic leukemia.

Longitudinal study and evaluation of clinical parameters with an influence on T-cell neogenesis

To determine the influence of transplant-related factors on T-cell neogenesis in more detail, we prospectively studied 31 patients in a longitudinal study. The median age of this group was 39 (range, 18-59) years. The median follow-up of the whole cohort after transplantation was 558 (range, 250 - 1231) days. We analyzed the impact of some pre-transplant conditions – age, T-cell depletion of the graft, intensity of the conditioning regimen, and the inclusion of total body irradiation – as well as the post-transplant events acute and chronic GvHD. The endpoint of the analysis was the achievement of normal age-adjusted sjTREC, βTREC and TF values during the observation period. Nine patients dropped out of the study due to early relapse or transplant-related mortality. Twenty-two patients were available for the final analysis with a sufficient number of samples (n=4-11). Of these 22 patients, eight (36%) reached normal sjTREC, βTREC and TF levels, whereas 14 did not. The median time to achieve age-adjusted normal levels within this group was 391 days and 240 days for sjTREC and βTREC, respectively. Of the clinical events evaluated in univariate analysis, acute GvHD had a statistically significant impact on βTREC recovery, (P=0.046) and especially on the TF (P=0.003) by Fisher's exact test. The influence on the achievement of a physiological reconstitution type and normal sjTREC was of borderline significance (P=0.05). Patients suffering from acute GvHD showed a rapid decline of βTREC, followed by low sjTREC production which generally did not reach normal values. In some patients, we could detect no sjTREC production for more than 1 year.

With respect to other clinical factors investigated, we observed a trend (P=0.08) towards the achievement of physiological reconstitution and higher sjTREC production in patients receiving reduced intensity conditioning. The influence of reduced intensity conditioning on βTREC (P=0.026) and the TF (P=0.032) was statistically significant. T-cell depletion or total body irradiation did not have a significant influence on TREC production. Age was not a significant factor for the achievement of normal TREC when the TREC target values were corrected for age (P=1.0) (Table 2).

The kinetics of TREC recovery in individual patients could be grouped into three patterns (Figure 4). The first group (group A, n=8) included patients reaching age-adjusted normal values for each of the three values within 2 years. The second group (group B, n=11) was characterized by low βTREC and low to moderate sjTREC resulting in a sj/β-TREC ratio close to normal. The pattern observed in group B was very similar to that observed in patients with acute GvHD and most patients in this group had clinically manifest acute GvHD at some time after transplantation. In the last subgroup (group C, n=3) neither sjTREC nor βTREC were detectable during the observation period.

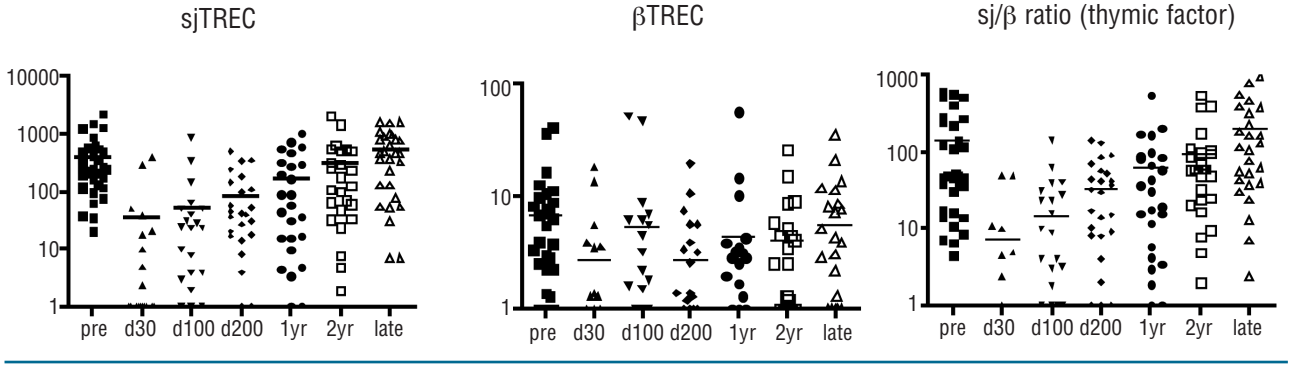

Figure 3. Reconstitution kinetics of TREC and the thymic factor. Pooled data from the 66 patients in the cross-sectional study. The values are given as copy numbers of the respective target gene normalized to 10^5 $CD3^+$ cells. All sjTREC values were measured in duplicate, whereas the βTREC numbers were measured in triplicate. Each point is the mean value of the multiple measurements of the respective sample.

Determination of the thymic output by immunophenotypic measurement of thymic naïve cells

Although our method for measuring RTE production appears to be informative about intrathymic events, it does not provide access to viable RTE for functional analysis. Several recent publications have provided evidence that the number of $CD3^+CD4^+CD45RA^+CD31^+$ T cells in the peripheral blood, the so-called thymic naïve population, correlates well with the number of sjTREC and can, therefore, be considered a valid measure of RTE production.[14,17] In a first step, we aimed to validate the published results. We determined the number of T cells with the latter phenotype in 15 healthy volunteers aged 24 to 92 years old and normalized this value to 10^5 $CD3^+$ cells. We found a significant decrease of thymic naïve cells with age

Table 2. Relation of risk factors and immunological end-points. Univariate analysis for different end-point/risk factor combinations based on contingency tables and two-sided Fisher's exact tests. The listed *P* values reflect the probability that one of the risk factors has influence on the respective end-point.

End-point vs risk factor	AGVHD	AGVHD or CGVHD	TCD	TBI	RIC	AGE
Physiological reconstitution	0.05	0.19	0.66	0.35	0.08	1.0
sjTREC	0.05	0.19	0.66	0.35	0.08	1.0
βTREC	0.046	0.079	0.39	0.33	0.026	0.68
Thymic factor	0.003	0.01	1.0	0.35	0.031	0.22

*AGVHD: acute graft-*versus*-host disease; CGVHD: chronic graft-*versus*-host disease; TCD: T-cell depletion by either* ex vivo *CD34 positive selection or the application of alemtuzumab; TBI: total body irradiation 4 or 12 Gy; RIC: reduced intensity conditioning. The term physiological reconstitution describes the achievement of age-adjusted normal sjTREC and βTREC values as well as a normal thymic factor during the post-transplant course.*

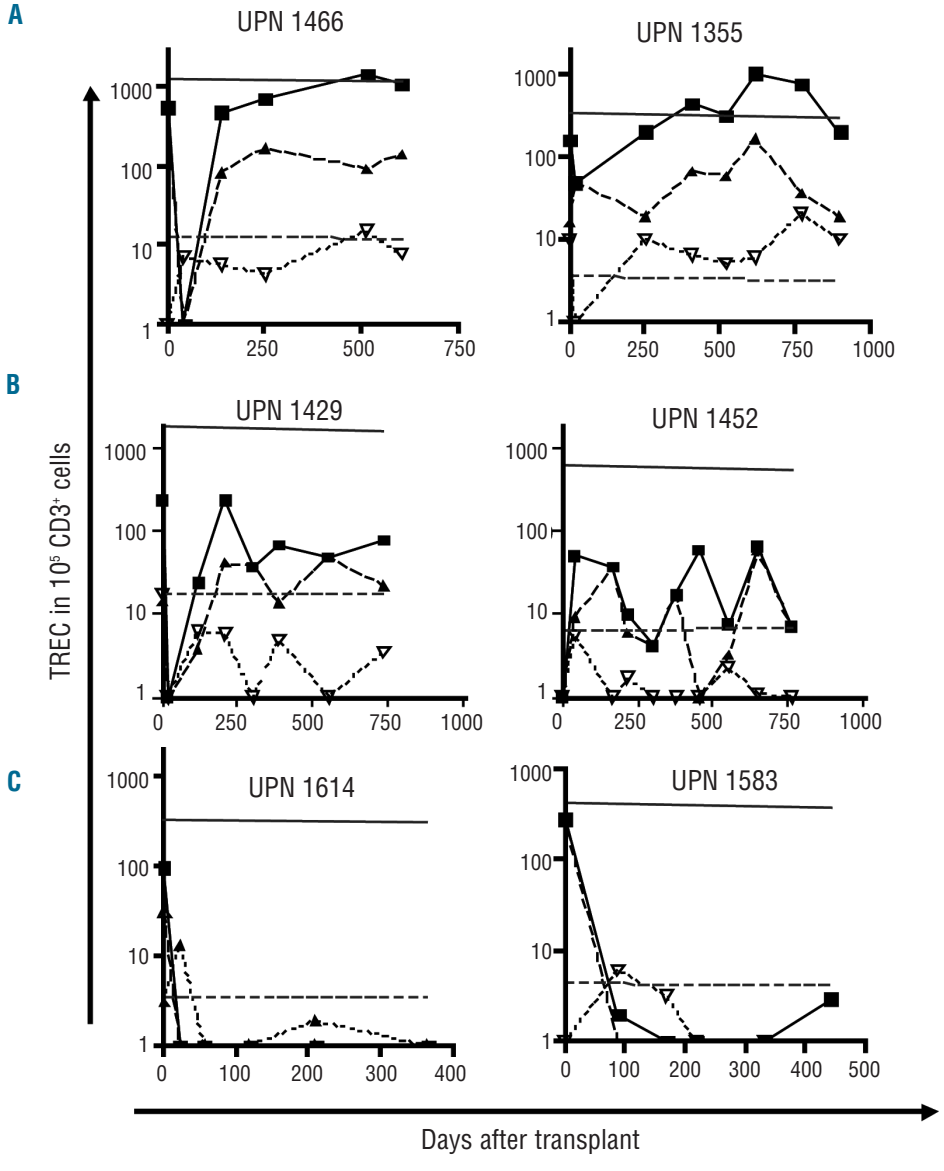

Figure 4. (A, B, C), Three different reconstitution patterns corresponding to groups A, B and C respectively described in the text. The diagram includes sjTREC (filled squares) and βTREC (open triangles) as well as the thymic factor (filled triangles). The horizontal solid line represents the age-adjusted normal values for sjTREC, while the horizontal dotted line represents the age-adjusted normal values of βTREC. Please note that the age-adjusted TREC standard values decrease as a function of time.

(*P*=0.028, Spearman's coefficient, r=-0.56), and there was a good correlation between the number of thymic naïve cells and the number of sjTREC (r=0.82). In a sequential subgating strategy employing six-color immunophenotyping, we then analyzed the proliferative activity within the thymic naïve cell population (R2 gate) by Ki-67 expression (Figure 1). In healthy volunteers the mean proliferation rate in this cell population was 0.36% (0-1.3%).

To assess the proliferative activity within the thymic naïve cell population in a lymphopenic setting we analyzed blood samples from 11 patients at various time points after allogeneic stem cell transplantation. Compared to samples from the healthy volunteers, the samples from these patients had a significantly higher proliferation rate within the thymic naïve population (t-test; *P*=0.0003); the mean of the peak proliferation rate in patients was 14.6% (1.8-50%).

When we analyzed the post-transplant kinetics of the proliferation rate in individual patients there appeared to be an inverse correlation with the severity of the lymphopenia and with the amount of T-cell neogenesis in the thymus, as measured by TREC analysis (*data not shown*). Unfortunately the number of patients and time points studied were not sufficient to demonstrate statistical significance.

Discussion

The primary goal of the present study was to establish a simplified method for measuring the TF, and to evaluate its usefulness in analyzing thymic events affecting T-cell neogenesis after allogeneic stem cell transplantation. Compared with the original method published by Dion *et al.*[25] our method requires a much lower number of PCR and thus the time and costs of the procedure are considerably reduced. Our modification does, however, result in the loss of the ability to measure T-cell receptor diversity.

The absolute numbers of sjTREC and βTREC and thus the TF we measured in healthy volunteers are close to those reported using the original method.[7,25] We also observed an inverse correlation between age and the TF, as previously reported. In contrast to the previous publications, however, the age-related drop in the thymic factor was not simply due to the reduced production of sjTREC but was associated with a lower number of βTREC as well.[7,25] The most likely explanation for this difference is the extended age range of the group of healthy volunteers we investigated. In the two previous reports, the upper age limit of the subjects investigated was 70 years, whereas our oldest volunteer was 92 years old. When the regression trend line was calculated only for patients aged up to 55 years (Figure 2C) separately, the line had a much slower decline, consistent with previous reports. Unexpectedly, βTREC production was significantly lower in the older age group than in the younger group. It was not, however, possible to determine the starting point of the βTREC decline since we did not analyze enough patients in the different age ranges. Nevertheless, we feel that the previous interpretation that the age-related decline in T-cell neogenesis is solely due to the reduced proliferation of intrathymic T cell progenitors is incomplete. Our data suggest that beyond the age of 70 there may be an impairment of T-cell neogenesis prior to the initiation of DβJβ – rearrangement which normally occurs in very immature T-cell progenitors.[25] This could be related to the well-documented age-related decline in lymphoid committed and/or thymus-seeding progenitors in both mouse and man.[29-31]

Our investigation of patients prior to conditioning revealed a pronounced deficit in T-cell production capacity compared with that in age-matched controls. We found a severe deficit in both sjTREC and βTREC suggesting that chemotherapy and/or radiotherapy affect both the intrathymic proliferation of T-cell progenitors and possibly the supply of thymus-seeding progenitors from the bone marrow. Given the cross-sectional nature of this analysis it was not possible to identify patterns of damage associated with different types of induction chemotherapy.

In our cross-sectional study with recovery of pre-conditioning TREC levels as the endpoint we observed a dramatic reduction of sjTREC early after transplantation followed by a slow and incomplete recovery over a period of 2 years with the number of βTREC remaining essentially stable. Age appeared to have a major impact on the speed of recovery after hematopoietic cell transplantation. This suggests that deficient intrathymic proliferation of immature T-cell progenitors is responsible for the reduced production of RTE. This would be consistent with the findings of published mouse studies. In the mouse, age, conditioning and GvHD appear to impair thymic neogenesis in the same way. These factors result in damage to the highly proliferative thymic epithelial cells[32] which causes reduced provision of intrathymic interleukin 7 and reduced proliferation of immature T-cell progenitors.[33,34]

Our ability to identify factors influencing T-cell neogenesis in our longitudinal study was limited by the sample size and was therefore restricted to performing univariate analysis. The development of acute GvHD had the statistically most significant impact on the probability of achieving a normal age-adjusted TF (*P*=0.003) after transplantation. The impact of GvHD was profound even though none of our patients developed more than grade II acute GvHD. This suggests that T-cell neogenesis is extraordinarily sensitive to GvHD. The intensity of conditioning and the degree of thymic damage prior to conditioning were of borderline significance. Age had no impact, which was not surprising since we used an age-adjusted endpoint.

The potential power of our method can be most clearly demonstrated by the fact that we were able to identify three different patterns of sjTREC and βTREC recovery following hematopoietic cell transplantation. Approximately 35% of patients (group A) showed the pattern described in the cross-sectional study implying that reduced intrathymic progenitor cell proliferation, presumably due to reduced interleukin-7 production by damaged thymic epithelial cells, is the predominant problem. If confirmed, this observation would have potential therapeutic consequences given that treatment with keratinocyte growth factor (KGF, Palifermin) and/or androgen blockade might protect the vulnerable thymic epithelial cells against damage and or accelerate their recovery.[33,35] The first clinical trials testing this hypothesis are ongoing,[36] and our method of measuring the TF might be helpful in monitoring the response to therapy. Approximately 50% of patients showed quite a different pattern of recovery with an almost complete lack of βTREC being the major defect (group B). This pattern of damage is closely associated with acute GvHD, and a French group made the same

observation using the original method for determining the TF.[26] This pattern suggests a block of T-cell neogenesis at a very early stage of development, i.e., the stage of the early thymic progenitors[9,25] or possibly even the thymus-seeding progenitors in the peripheral blood or bone marrow.[37,38] There is good experimental evidence that acute GvHD results in early damage of the bone marrow stromal niches and a subsequent loss of early lymphoid progenitors.[38] There is also growing evidence from mouse models of hematopoietic cell transplantation that the availability of thymus-seeding progenitors could be the rate-limiting factor for T-cell neogenesis, independently of allogeneic effects. These observations imply that this pattern of damage might be overcome by the provision of lymphoid progenitors or thymus-seeding progenitors. In the remaining 15% of patients (group C) neither sjTREC nor βTREC could be detected suggesting a global pre-thymic and thymic defect in T-cell neogenesis, which presumably could only be overcome by combining several different therapeutic interventions. Given the potential therapeutic consequences, additional studies in larger cohorts of patients are required to assess the value of our method further.

The second focus of this paper was on the dynamics of the post-thymic compartment, i.e., the RTE compartment. In the mouse system there is clear evidence that RTE are immature cells and undergo further maturation and proliferation in the niches of secondary lymphoid organs. The information on human RTE is limited because until recently there were no markers available to distinguish RTE from naïve T cells. The availability of CD31 and/or PTK as surface markers for RTE has provided evidence that human RTE are indeed a functionally immature subpopulation of T cells with a reduced response to stimulation by cytokines and antigen.[13,17]

Since the absolute number of thymic naïve $CD4^+$ T cells in the peripheral blood of healthy volunteers is low, it was vital to validate the usefulness of CD31 as a marker for RTE. As previously demonstrated by others,[17,19] the number of $CD3^+CD4^+CD45RA^+CD31^+$ T cells was inversely correlated with age and showed a good correlation with the sjTREC. The concomitant study of Ki67 expression revealed a very low amount of proliferation in the thymic naïve subpopulation. These results are consistent with results recently published by Matsuoko *et al.*[16] and with those of previous studies that had provided indirect evidence of proliferative activity in the $CD31^+$ subpopulation of naïve $CD4^+$ T cells by demonstrating a declining sjTREC content and a variable decrease of telomere length with age.[17,19,39] When we performed the same analysis on RTE from patients early after transplantation, we found a significantly higher proliferation rate of RTE in patients than in healthy controls. Matsuoko *et al.*[16] also reported a higher proliferation rate of naïve T cells in a cohort of patients after allogeneic hematopoietic stem cell transplantation compared to healthy donors, although this effect was more pronounced in the Treg population than in conventional T cells. By subdividing the $CD31^+$ cells in $CD45RA^+$ and $CD45RA^-$ cells Matsuoka *et al.* found only very low proliferation in the $CD45RA^+$ $CD31^+$ Treg cell population. This might be explained by their exclusive use of T-repleted grafts compared to our employment of T-cell depleted grafts in over 70% of the patients leading to a much greater degree of lymphopenia immediately after transplantation.

An enhanced proliferation rate for naïve T cells after allogeneic hematopoietic stem cell transplantation has also been reported by other groups.[16,23,40] The proliferation in the RTE pool could be due to cytokine-mediated homeostatic proliferation given the fact that there is good *in vitro* evidence that $CD3^+CD4^+CD45RA^+CD31^+$ RTE can proliferate in response to cytokines alone and that such proliferation is not associated with down-regulation of CD31.[41,42] One candidate cytokine is interleukin-7 which, when given to cancer patients in a recent phase I study, induced a preferential expansion of $CD3^+CD4^+CD45RA^+CD31^+$ RTE although it was not entirely clear whether the expansion of these cells occurred in the thymus or in peripheral lymphoid tissues.[43] In the small cohort of patients with longitudinal data we observed intriguing fluctuations of proliferative activity. Identification of the factors responsible for these fluctuations will require further study. Overall our data suggest that the combined application of these two approaches has the potential to further improve our understanding of the thymic and post-thymic phases of T-cell neogenesis after allogeneic hematopoietic cell transplantation.

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